Universidade de Aveiro Departamento de Biologia
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Estabilidade Conformacional da Proteinase Aspártica – Arquétipo, Pepsina A

Conformational Stability of the Archetypal Aspartic Proteinase, Pepsin A

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Professora Doutora Marlene Maria Tourais de Barros, Professora Associada com Agregação do Centro Regional das Beiras da Universidade Católica Portuguesa, e do Professor Doutor Euclides Manuel Vieira Pires, Professor Associado do Departamento de Bioquímica da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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A meus Pais e Irmã

o júri

presidente

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Errata da Dissertação

“Estabilidade Conformacional da Proteinase Aspártica - Arquétipo, Pepsina A”

Na primeira página, deveria ter-se incluído:

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palavras-chave

Pepsina A; Acetonitrilo; Hidrocloreto de Guanidina; Ureia; Temperatura; Desnaturação de Proteínas; Estados Conformacionais Não-Nativos; Estabilidade Conformacional.

resumo

A pepsina A tem sido considerada um protótipo do grupo das proteinases aspárticas, cuja relevância ao nível da patofisiologia humana tem estimulado intensa pesquisa. As propriedades moleculares e catalíticas da pepsina A começaram a ser exploradas há cerca de um século. Contudo, a informação existente sobre a estabilidade conformacional desta proteína em condições nativas é insuficiente. Elucidação dos factores responsáveis pela manutenção do estado conformacional naturalmente enrolado e activo de proteínas é vital para estabelecer estratégias de modulação da estabilidade. Com o objectivo de obter novos conhecimentos acerca das bases da estabilidade conformacional da pepsina A, foi adoptada uma abordagem que consistiu na indução e monitorização da desnaturação da proteína no seu estado naturalmente enrolado. A severidade das condições ambientais foi gradualmente agravada por adição de desnaturantes e / ou modificação de propriedades físicas e químicas do sistema proteína-solvente. Após uma fase de incubação nas condições desnaturantes, com a duração típica de 1 h, mudanças nas propriedades espectrais, no comportamento hidrodinâmico, na estabilidade térmica e na actividade peptidolítica da pepsina A de suíno foram avaliadas. O solvente orgânico, acetonitrilo, induziu destruição não-cooperativa dos arranjos secundário e terciário desta proteína. Sugere-se que a drástica debilitação das estruturas de hidratação pelo acetonitrilo terá impelido o desenrolamento global. Adicionalmente, a pepsina A revelou-se particularmente resistente à acção desordenante de dois agentes caotrópicos clássicos, o hidrocloreto de guanidina (GdnHCl) e a ureia. Um estado intermediário estável foi identificado durante a reacção de desenrolamento da proteína nativa induzida pelo GdnHCl. A adição de acetonitrilo a uma molaridade intermédia permitiu a identificação de um diferente estado parcialmente desenrolado na presença de GdnHCl a concentrações pré-transicionais. Dados espectroscópicos indiciam promoção de ordem local a nível do esqueleto polipeptídico pela ureia a baixas e altas molaridades. Indução de extenso desenrolamento da proteína pela ureia requereu concentrações quase saturantes deste desnaturante e períodos de exposição de duração superior a 1 h. Foram detectados fenómenos de aparente estabilização, quando a fase de pré-incubação da proteína em ambos os agentes caotrópicos a baixas molaridades foi prolongada para 3 dias. A pepsina A no seu estado nativo revelou-se bastante termoestável. Contudo, 1 h de exposição da proteína, antes do seu aquecimento, a qualquer dos três desnaturantes a concentrações pré-transicionais foi suficiente para aumentar a termolabilidade da proteína. Contribuições de interacções hidrofóbicas e estruturas de solvatação para a estabilidade conformacional da pepsina A no seu estado biologicamente activo são discutidas nesta dissertação.

keywords

Pepsin A; Acetonitrile; Guanidine Hydrochloride; Urea; Temperature; Protein Denaturation; Non-Native Conformational States; Conformational Stability.

abstract

Pepsin A has been considered a prototype of the group of aspartic proteinases, whose significance in human pathophysiology has galvanized intensive research. Molecular and catalytic properties of pepsin A have been explored for about a century. However, there is only fragmentary data on the conformational stability of this protein under native conditions. Elucidation of the factors accountable for the maintenance of the natively ordered and active conformational state of a protein is vital for engendering strategies for tailoring stability. In order to gain new insights into the scaffold of the conformational stability of pepsin A, an approach based on induction and monitoring of denaturation of the natively folded protein was adopted throughout the investigation reported herein. Environmental stringency was gradually increased by adding denaturants and / or modifying chemical and physical properties of the protein-solvent system. After a period of incubation in the denaturing conditions, which lasted typically 1 h, changes in spectral properties, hydrodynamic behaviour, thermal stability and peptidolytic activity of porcine pepsin A were appraised. The organic solvent, acetonitrile, was found to induce non-cooperative destruction of secondary and tertiary arrangements of this protein. Drastic debilitation of hydration structures by acetonitrile is suggested to have triggered global unfolding. In addition, pepsin A revealed to be particularly resistant to the disordering action of two classic chaotropes, guanidine hydrochloride (GdnHCl) and urea. A stable, intermediary state could be identified in the pathway of GdnHCl-induced unfolding of native pepsin A. Upon addition of acetonitrile at an intermediary molarity, a different partially unfolded state could be identified in the presence of GdnHCl at pre-transitional concentrations. It is apparent from spectroscopic data that urea promoted local backbone order at low and high molarities. And, periods of exposure longer than 1 h to urea at near-saturating concentrations were required to onset gross unfolding by this denaturant. Phenomena of apparent stabilization were detected when submission of the protein to both chaotropes at low molarities was prolonged to 3 days. Furthermore, pepsin A in its native state was noted to be rather thermostable. However, 1 h - incubation at pre - transitional concentrations of any denaturant prior to heating caused a raise in the protein thermolability. Contributions from hydrophobic interactions and solvation structures to the conformational stability of pepsin A in its biologically active state are discussed in this dissertation.

Cover Illustration:

Transparent molecular surface of porcine pepsin A (PDB code *4pep*) with the backbone rendered as a ribbon. This figure was generated by *GRASS* (*Graphical Representation and Analysis of Structure Server* available at http://trantor.bioc.columbia.edu/cgi-bin/GRASS/surfserv_enter.cgi) (Nayal, *et al.*, 1999).

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SYMBOLS AND ABBREVIATIONS

θ – Ellipticity

$\Delta\lambda_{max}$ – Difference between the Fluorescence Emission Maxima of Model Compound and Protein

$[\theta]_{MRW}$ – Mean Residue Ellipticity

ΔH_{cal} – Calorimetric Enthalpy

ΔH_{vH} – van't Hoff Enthalpy

λ_{max} – Wavelength of Maximum Fluorescence Emission

ε_y – Extinction Coefficient

A – Absorbance

ANS – 8-Aniline-1-Naphthalenesulfonate

ATP – Adenosine 5' Triphosphate

BCA – Bicinchoninic Acid

BSA – Bovine Serum Albumin

C – Concentration (M)

c – Concentration (mg/mL)

C-Terminus – Free α-Carboxyl Group (at one end of a protein)

CE – Combinatorial Extension

DAPI – 4', 6-Diamidino-2-Phenylindole

DMSO – Dimethyl Sulphoxide

EC – Enzyme Commission

F – Folded State

FPLC – Fast Protein Liquid Chromatography

GdnHCl – Guanidine Hydrochloride

GRASS – Graphical Representation and Analysis of Structure Server

HIV-1 – Human Immunodeficiency Virus Type 1

HPLC – High Performance Liquid Chromatography

HT – High Voltage

I – Intermediary State

I_p – Alkaline-Denatured State of Pepsin

K_{av} – Gel-Phase Distribution Coefficient

l – Path Length

Me – Methyl

M_r – Relative Molecular Weight (or Mass)

MRW – Mean Residue Weight (or Mass)

N-Terminus – Free α-Amino Group (at one end of a protein)

NATA – N-Acetyl-L-Tryptophanamide

Nitro – NO_2

P – Product

PAGE – Polyacrylamide Gel Electrophoresis

PDB – Protein Data Bank

pK_a – Acid Dissociation Constant

PSI – Plant-Specific Insert

rpm – rotations per minute

S – Substrate

SASA – Solvent Accessible Surface Area

SCOP – Structural Classification of Proteins

SDS – Sodium Dodecyl Sulphate

Selcon3 – Self-Consistent Method for Protein Circular Dichroism Analysis,Version 3

TEMED – N, N, N', N'-Tetramethylene-Ethylenediamine

TFA – Trifluoroacetic Acid

Tris – 2-Hydroxymethyl-2-Methyl-1, 3-Propanediol

U – Unfolded State

UV – Ultraviolet

V_0 – Column Void Volume

V_e – Elution Volume

V_t – Total Bed Volume

NOTATION AND NUMBERING

Schechter and Berger notation will be adopted to designate the subsites of a proteinase (….S_3, S_2, S_1 and S_1', S_2', S_3'….), and the corresponding amino acid residues of a substrate (…P_3, P_2, P_1 and P_1', P_2', P_3'….), on the N- and C-terminal sides of the scissile peptide bond ($P_1 – P_1$') (Schechter & Berger, 1967).

Standard single- or three-letter codes will be used for amino acid residues whenever appropriate.

Unless otherwise stated, amino acid sequence numbering for porcine pepsin A (Sielecki, *et al.*, 1990) will be assumed throughout the present dissertation. The suffix 'p' is used to denote residues in the prosegment of porcine pepsinogen A.

"In the end, this is all about finding out how and why nature contradicts universal entropy in order to create order."

ANONYMOUS

CHAPTER I

INTRODUCTION

The importance of proteins, primarily as macromolecules essential for the development and maintenance of life, is undeniable. Besides, their properties are so valuable that proteins have acquired a notable status as polymers with industrial and pharmaceutical applications for long. Indeed, nowadays proteins are used as catalysts, materials and therapeutic agents.

The development of *protein science* has been significantly fed by the challenge of devising strategies for modulation of folding, conformation, stability and activity of proteins. The successful attainment of these aims implies at least: (i) clarification of relationships between protein conformation and biological function (*i. e.* of the *classic structural biology paradigm*); (ii) decipherment of the folding code; and (iii) thorough establishment of the physical and chemical principles assuring the maintenance of the native and biologically active conformational state of a protein. Postulates regarding these issues have been pursued with great energy by molecular biologists and biochemists. The legacy of an increasing number of protein sequences available through the genome projects (Fulton, *et al.*, 2005; Saghatelian & Cravatt, 2005) and growing evidence for the implication of protein misfolding and aggregation in many diseases (Ingelsson, *et al.*, 2005; Kransnoslobodtsev, *et al.*, 2005; Sambamurti, *et al.*, 2006) are recent incentives.

In this vein, *protein engineering* is one of the strategies mostly adopted. A complementary approach is based on the influence of the chemical composition and physical characteristics of the surrounding environment on protein conformations. These methods have contributed to the inference of a number of requirements for specific functional, structural and biophysical properties of proteins. Increasing data in this direction have been reinforcing aspirations of theoreticians and experimentalists who are aiming to effectively enhance folding efficiency and fine-tune activity and stability of proteins in view of specific purposes.

In the end, the ultimate and ambitious goal of protein science, the rational design of *de novo* proteins, will hopefully be fulfilled, and many avenues leading towards useful technological applications of proteins will be improved or opened. From medicine and nutrition to industry, a wide range of fields are envisaged to be touched by the unpaired potential of the arising benefits.

1. *PROTEIN DENATURATION: INSIGHTS INTO FOLDING AND CONFORMATIONAL STABILITY*

Supervision of induced order-to-disorder transitions in proteins, eventually followed by reconstitution of native-like conditions, and characterization of implicated conformational states may yield a wealth of information about the determinants (forces and molecular characteristics) of conformational stability, as well as shed some light on the prime factors driving folding, unfolding, misfolding and aggregation. Provided that care is taken when performing extrapolations, the presence of folding intermediates might be predicted and folding pathways might be decoded. Ultimately, a more detailed picture of the molecular events and energetics implied in any transition between conformational states might be sketched (*vide*, for example, the following references: Bhavesh, *et al.*, 2003; Caflisch & Karplus, 1994; Finkelstein, 1997; Grant, *et al.*, 1992; Pace, *et al.,* 1990b; Stapelfeldt & Skibsted, 1999).

It is the goal of this subchapter to put current knowledge on protein denaturation into a comprehensive perspective, and to emphasize its potentials as an individual or complementary methodology for understanding folding and assessing conformational stability. Firstly, we should concentrate on the diversity of denatured states and unfolding pathways. Edifying information on protein folding obtainable from the employment of protein denaturation and renaturation techniques will also be outlined. Lastly, marginal conformational stability of protein native states will be explored in terms of its physical and molecular foundations, as well as biological rationales.

Before proceeding, it urges to clarify that the following discussion implies the classic axiom, by which a folded conformational state is demanded for adequate protein function. Furthermore, even though many principles lying beneath conformation, stability and unfolding / folding reactions are similar for different architectural categories of proteins, the prime targets of the subsequent description are globular proteins.

A. *Theoretical Framework for Protein Denaturation*

Modifications in pressure, pH, ionic strength or temperature of the surrounding environment (Arnold, *et al.*, 1996; Bai, *et al.*, 1998; Jayaraman, *et al.*, 2006; Stapelfeldt & Skibsted, 1999; Stigter, *et al.*, 1991), or addition of ligands, detergents, chaotropes, reducing agents or organic solvents to the protein-solvent system (Akhtar, *et al.*, 2002; Bettati, *et al.*, 2000; Li, *et al.*, 1995; Mozhaev, *et al.*, 1989; Otzen, 2002; Simon, *et al.*, 2001) usually alter the delicate balance of forces accountable for preservation of the biologically active and native conformational state of a protein. The term '*denaturation*' refers to any process by which a protein is perturbed. At most times, denaturation is viewed as an order-to-disorder conformational transition, whereby secondary, tertiary, and quaternary structures of a protein are disrupted; even though it

does not necessarily lead to complete unfolding. Instead, structural disarray is sometimes restricted to definite regions within the biomacromolecule, insomuch that some interactions might remain unchanged or even be intensified, and new ones might be formed. And, thus, distinct non-native states with intermediary properties between those of the natively folded and the fully unfolded states might appear (Halle, *et al.*, 2005; Uversky, 2002a). A reduction in or a complete loss of activity often accompanies protein disordering transitions (Chen, *et al.*, 2003 ; Ternström, *et al.*, 2005; Wei, *et al.*, 2006).

By virtue of its primacy in elucidating principles governing protein folding, conformation and stability, protein denaturation has become a relevant research discipline (Fleming & Rose, 2005). Nowadays, conformational transitions can be followed step by step in great detail. A plethora of experimental techniques, including, for instance, circular dichroism or fluorescence spectroscopy (Rodger & Ismail, 2000; Szabo, 2000), have afforded delineation of folding and unfolding pathways, and physicochemical characterization of intermediary and final states implicated therein. The usefulness of theoretical methods in providing insights into these fields has also been recognized. Application of molecular dynamics simulations to protein denaturation using detailed atomic models for protein solvent is an evocative example (Brooks, 1998; Caflisch & Karplus, 1994). Merging of experimental and theoretical approaches is being implemented, in order to give atomic-level descriptions and predictions of protein conformational transitions (Fersht & Daggett, 2002).

A.1. *Phenomenological Aspects*

A.1.1. *Modes of Unfolding*

Global unfolding might be considered an extreme case of denaturation, for it results in a largely extended polypeptide chain characterized by the absence of any (or almost any) ordered structural elements. A second mode of unfolding has been distinguished, the so-called *local unfolding*, which corresponds to structural unravelling of confined regions within a protein (Neupert, *et al.*, 2005).

Phenomena of local unfolding occur even in a protein under native conditions, where they are prompted by everlasting thermal fluctuations (Gaume, *et al.*, 1998; Neupert, *et al.*, 2005; Zhuang, *et al.*, 2000). Conditions could occur in which the free energy barrier between folded and unfolded states is not surmounted, and refolding within a short period of time follows as a consequence (Dokholyan, *et al.*, 2000). Otherwise, entrapment of local unfolded segments in a protein might occur due to, for instance, binding of denaturants (Zhuang, *et al.*, 2000). Once these protein segments are trapped, unfolding will proceed, possibly leading to a completely unfolded state within milliseconds to minutes (Neupert, *et al.*, 2005). Besides prompting global structural disarray, local unfolding events may as well facilitate aggregation (DeMarco & Daggett, 2005) and proteolysis processes (Fontana, *et al.*, 1997; Vriend, *et al.*, 1998).

A.1.2. *Reversibility* versus *Irreversibility*

A *reversible* denaturation process entails total recovery of the starting state, in conformational and functional terms, through the reverse reaction (Ahern & Klibanov, 1988; Volkin & Klibanov, 1991). In the main, reversibility of protein denaturation depends on the environmental conditions and on the period of exposure to perturbants (Naik & Huang, 2004).

Several physical and chemical changes occurring upon an order-to-disorder transition might produce alterations in the folding properties of the polypeptide chain and hamper the back-reaction. Structural perturbations in the protein facilitate exposure of residues amenable to chemical modifications or peptide bonds liable to cleavage, hitherto more or less protected in the folded conformational state, thereby threatening the integrity of the polypeptide chain. A long list of covalent deteriorative mechanisms may be responsible, or co-responsible, for irreversibility of protein disordering processes, including, for example, hydrolysis of peptide bonds at the carboxyl terminus of aspartic residues at acidic pH, deamidation of amine residues, destruction of cystines, autolysis or degradation by exogenous proteinases, among others (Ahern & Klibanov, 1988; Mozhaev & Martinek, 1982; Tomazic & Klibanov, 1988; Vieille & Zeikus, 2001; Volkin & Klibanov, 1990; Volkin & Klibanov, 1991). Denaturation irreversibility is also often ascribed to intermolecular associations resulting in *aggregated states* (Ahern & Klibanov, 1988; Pace, 1986; Volkin & Klibanov, 1991). Unfolding leads to increased exposure of non-polar groups hitherto buried, which may cause the denatured protein to self-associate. These associations may either occur via hydrophobic interactions, so that the unfavourable contact between hydrophobic amino acid residues and water is minimized (Patro & Przybycien, 1994), or via chemical reactions, mainly through the formation of intermolecular disulphide bridges (Volkin & Klibanov, 1990).

A.2. *Denatured States*

Any incorrectly folded or ill-defined, disordered conformational state of a polypeptide chain might be simplistically referred to as a *denatured state* (Hamada & Goto, 2005; Uversky, 2002a). In general, each denatured state should be viewed as a collection of diverse interconverting conformations larger than the corresponding natively folded-state ensemble, due to higher structural heterogeneity (Fersht & Daggett, 2002; Shortle, 1996; Shortle, *et al.*, 1998). In the past few years, substantial attention and effort have been devoted to the description of non-native states. Nevertheless, experimental endeavours are often threatened by technical difficulties arising from, for example, their insolubility or high flexibility (Dill & Shortle, 1991; Shortle, 1996). Therefore, fine structural details on denatured states are yet to be worked out.

A.2.1. *Structural Diversity*

Herein, a brief note on the terminology adopted in the scope of denatured states, throughout the

current literary review and for the remainder of this dissertation, shall be introduced. A semantic scheme was formalized mainly on the basis of reconciliation between different classification systems of conformational states assumed by several authors (Cooper, 1999; Uversky, 2002a). And, it relies on two central premises: (i) as aforementioned, any disordered or incorrectly folded conformation, different from the native conformation, is a denatured conformation; and (ii) an unfolded conformation is an unstructured conformation. In face of this, denatured states can be assorted into structurally diverse subsets, *viz.*, *molten globule states*, *misfolded states* and *unfolded states*.

A description of molten globule states will be provided elsewhere. Misfolded states are produced in the course of protein folding, and result from entrapment of the folding molecule in aberrant conformations. These, however, might exhibit, to some extent, similarities to corresponding native folds (Cooper, 1999; Dobson, 2005; Privalov, 1996).

A.2.1.1. *Unfolded States*

Each unfolded state of a protein comprises a particularly large and heterogeneous ensemble of many open, irregular and fluctuating conformational substates (Bachmann & Kiefhaber, 2005; Cooper, 1999; Cooper, 2000a; Fleming & Rose, 2005; Ohnishi & Shortle, 2003). An unfolded form is fluid-like and the bonds of its amino acid side chains rotate quite freely (Haynie, 2001). Despite its high degree of unstructureness, an unfolded state may exhibit intramolecular hydrophobic interactions and specific hydrogen bonds. Non-covalent interactions between residues are, however, expected to be neither stable nor abundant (Creighton, 1990; Pace, *et al.*, 1996). Owing to predominant exposure of protein groups to solvent, many intramolecular interactions within folded conformations are substituted by protein-solvent interactions in the unfolded state (Cooper, 2000a).

A more accurate definition of unfolded state should consider a set of conformational states characterized by any or almost any regular structure (Cooper, 1999; Uversky, 2002a), including *premolten globule states* (for further information, *vide infra*), *aggregates* and conformational states well-approximated by the *random coil* model.

A.2.1.1.1. *Random Coil*

Random coil is traditionally regarded as the ideal unfolded form for a protein. Apart from complete freedom of bond rotation (Fleming & Rose, 2005), the random coil model further assumes the absence of any local interactions along the polypeptide chain, and full exposure of all amino acid side chains (Haynie, 2001; Wirmer, *et al.*, 2005). Yet, features attributed to the random coil model are not perfectly recognizable in real unfolded conformational states of proteins. In a real random coil (or random coil - like conformation) some atoms of the polypeptide chain may be in close proximity with each other, so that significant steric effects, and consequently, restrictions on the local flexibility may actually take place (Creighton, 1990; Tanford,

1968; Uversky, 2002a). Besides some weak local interactions and few, if any, non-local interactions (Smith, *et al.*, 1996; Wirmer, *et al.*, 2005), random coil - like conformations have some tendency to display residual helicity (Creighton, *et al.*, 1996).

A.3. *Pathways of Protein Unfolding*

This subsection is intended to overview unfolding pathways recognized for several proteins. From this point on, the phrase 'unfolding transition' will refer to a structured-to-unstructured transition, whose end-point is an unfolded state.

A.3.1. *Two-State Unfolding and Cooperativity*

If non-covalent stabilizing interactions in a protein are so dependent on each other, insomuch that disruption of a small number of interactions triggers concurrent disruption of all the remaining non-covalent interactions upon protein unfolding; then the folded-to-unfolded transition is said to be *cooperative*. This would be tantamount to saying that there is little or no partial unfolding preceding the achievement of the unfolded state. Any conformational strain that renders a partially folded state unstable might contribute to cooperativity. Disruption of an internal hydrogen bond without supplying comparable hydrogen-bonding partners to the acceptor or donor, or sufficient separation of two non-polar surfaces, which will engage comparable interactions with other surfaces or with the solvent, are evocative examples (Creighton, 1990; Doster & Friedrich, 2005).

Cooperativity of protein unfolding entails the occurrence of only two significantly populated conformational species at equilibrium in the transition region: the folded (F) and the unfolded (U) macrostates (Cooper, 1999; Maki, *et al.*, 2005). Equilibrium *two-state unfolding transitions* are usually depicted by:

$$\mathrm{F} \underset{k_{\mathrm{folding}}}{\overset{k_{\mathrm{unfolding}}}{\longleftrightarrow}} \mathrm{U} \qquad (1.1),$$

where $k_{\mathrm{unfolding}}$ and k_{folding} stand for rate constants of the unfolding and folding reactions, respectively.

Apart from the folded and the unfolded macrostates, intermediary structures are always formed in single-step unfolding transitions (Genzor, *et al.*, 1996; Neet & Timm, 1994; Vaintraub & Morari, 2003). Nonetheless, such partially unfolded transient conformations are only infinitesimally populated along equilibrium all-or-none transitions, for they are energetically unstable under all conditions. And, thus, these intermediary forms are undetectable by experimental measurements (Creighton, 1990; Milne, *et al.*, 1999;

Vaintraub & Morari, 2003). The only species between the natively folded and the unfolded macrostates accessible to experimental study in a two-state unfolding reaction is the *transition state*. It is worth mentioning that the transition state is the species of the reaction pathway displaying the highest free energy. Such intermediate is unstable and corresponds to the folding core (Fersht & Daggett, 2002; Galzitskaya & Finkelstein, 1999). Transition states can be detected resorting to kinetic methods (Fersht & Daggett, 2002).

Unfolding of small, globular, monomeric proteins frequently conforms to the two-state model (Ganesh, *et al.*, 1997; Naik & Huang, 2004; Zolkiewski, *et al.*, 1996). Such model has also been applied to describe equilibrium, bimolecular unfolding processes of a group of dimers, in which the two stable macrostates consist of a folded dimer and an unfolded monomer (Mazzini, *et al.*, 2002; Neet & Timm, 1994).

A.3.2. *Multi-State Unfolding and Intermediary States*

At each step of a structured-to-unstructured transition, reduction or even complete extinction of non-covalent interactions may be restricted to part of the whole molecule. Thereby, overwhelmingly populated, metastable, partially folded forms, with intermediary properties between those of folded and completely unfolded states, may sometimes appear along the course of an unfolding reaction (Halle, *et al.*, 2005; Milne, *et al.*, 1999; Uversky, 2002a). *Intermediary states* have been detected by both equilibrium and kinetic methods (Jennings & Wright, 1993; Maki, *et al.*, 2005).

Unfolding transitions by which the initial folded state is converted into the final unfolded state via a series of discrete intermediary states, [I(i)], are designated equilibrium *multi-state unfolding transitions*, and are usually depicted by the following scheme (Makhatadze, 2005):

$$F \leftrightarrow I(1) \leftrightarrow I(2) \leftrightarrow \ldots \leftrightarrow I(n) \leftrightarrow U \qquad (1.2).$$

Proteins composed of multiple individual cooperative elements are amenable to stepwise unfolding. Such behaviour is frequently observed for multidomain (Ahmad, *et al.*, 2005; Akhtar & Bhakuni, 2003; Vlasov, *et al.*, 1996) and multimeric proteins (Duan & Nilsson, 2005; Hsieh, *et al.*, 2005; Mazzini, *et al.*, 2002; Mei, *et al.*, 2005).

A.3.2.1. *Molten Globule States*

Molten globule states have been observed in unfolding pathways of several globular proteins. These metastable intermediates have been found to accumulate under mild denaturing conditions, such as: (i) moderately low or high pH (Genzor, *et al.*, 1996; Kuwajima, 1996; Marcos, *et al.*, 2000; Morozova-Roche, *et al.*, 1997; Poklar, *et al.*, 1997); (ii) low concentrations of alcohols (Bychkova, *et al.*, 1996; Uversky, *et al.,* 1997); and (iii) mild concentrations of chaotropes (Kikuchi, *et al.*, 1998; Kuwajima, 1996; Mizuguchi, *et al.*, 1998; Uversky, 1993; van Dael, *et al.*, 1993). Nonetheless, molten globule states have also been detected

under high temperature (Maki, *et al.*, 2005) as well as under high pressure (Lassalle, *et al.*, 2003; Vidugiris & Royer, 1998).

General conformational and dynamical characteristics of a molten globule state are: (i) it is as nearly compact as the folded state (Bychkova & Ptitsyn, 1993; Liu, *et al.*, 2006; Vassilenko & Uversky, 2002); (ii) its internal fluctuations are presumed to be much larger as compared to fluctuations in the folded state (Vassilenko & Uversky, 2002); (iii) it generally has a significant amount of secondary structural elements, but lacks rigid tertiary structure (Bychkova & Ptitsyn, 1993; Sheshadri, *et al.*, 1999; Vassilenko & Uversky, 2002); (iv) it is stabilized by hydrophobic interactions, but its non-polar core is loosely packed (Lassalle, *et al.*, 2003; Vassilenko & Uversky, 2002).

Almost certainly, molten globule states are in conformational equilibrium with natively folded states in living cells. Aside from protein folding, it has been deemed that molten globule states actively participate in diverse biological processes, namely, protein aggregation, degradation and transport (Bychkova, *et al.*, 1988; Bychkova & Ptitsyn, 1993).

A.3.2.2. ***Premolten Globule States***

A premolten globule state is a distinct thermodynamic state, whose overall conformation is surmised to be intermediary between molten globule and random coil - like states. Besides the absence of a rigid tertiary structure, the secondary structure contents of a protein in the premolten globule state are approximately 50% of that of the corresponding ordered state. Notwithstanding the compactness of the premolten globule state is considerably lower relative to the molten globule or folded states; a protein in this conformational state is still more compact than a random coil - like state. It has been further ascertained that in the premolten globule state the protein does not exhibit a globular shape, but it is less extended than a random coil - like state (Uversky, 2002a; Uversky, 2002b). Reports on the occurrence of premolten globule states in unfolding pathways are still rare (for an example, consult the following reference: Uversky & Ptitsyn, 1996).

B. ***Protein Denaturation*** **in vivo**

Besides the benefits of incisive research on disordering transitions and implicated conformational states pinpointed earlier in this subchapter, additional advantages may be envisaged. Suffice it to say that subtle interconversion between alternative forms is often required for efficacious functioning of a protein *in vivo*. Deep investigations have inclusively demonstrated relationships between functional properties of a protein, and conformational features of, not only the natively folded state of its free form, but also altered conformational states induced by binding of ligands or by action of effectors. Therefore, the elucidation of structural details of alternate functional states, along with the understanding of energetic and molecular

transformations accompanying conformational conversions, grants edifying insights into *in vivo* processes promoted by proteins (Shortle, 1996).

Relevance of studies of protein denaturation *in vitro* is further reinforced by the pathophysiological significance of more severe order-to-disorder transitions in the cell. On one hand, 'accidental' denaturation events, whereby specific proteins or fragments change from non-toxic to toxic forms, occur and turn out to be key steps in the so-called *conformational diseases*. Prion disorders are illustrative examples (Ferreira & de Felice, 2001; Jackson, *et al.*, 1999). On the other hand, regulated reactions of full and partial unfolding have proved to be crucial in several steps of the life cycle of many proteins, such as dismantling of protein complexes, protein degradation by ATP-dependent proteases and protein translocation across membranes (Fersht & Daggett, 2002; Gaume, *et al.*, 1998; Huang, *et al.*, 1999; Matouschek, 2003; Matouschek & Bustamante, 2003; Neupert, *et al.*, 2005; Poklar, *et al.*, 1997). A brief allusion to the role of partially (un)folded states *in vivo* had already been made under the heading '*Molten Globule States*'. Notwithstanding mechanisms of protein denaturation within the cell are not yet fully understood, it is well-established that unfoldases play a role in catalysis of *in vivo* unfolding. Such biocatalysts are able to increase the speed of conformational transitions by interfering with the unfolding pathways of their substrates (Huang, *et al.*, 1999; Lee, *et al.*, 2001). The local structure encountered by the unfoldase as it follows the polypeptide chain of its substrate from the targeting signal is believed to determine the protein resistance against unfolding (Lee, *et al.*, 2001; Matouschek & Bustamante, 2003).

C. *Drawing Inferences about Protein Folding*

Thorough understanding of protein folding remains one of the major scientific contemporary challenges. Advances on this field have opened prospects of predicting protein folding reactions in the cell, and devising strategies to avoid and minimize protein misfolding, aggregation and instability, with implications for the combat of conformational diseases. Furthermore, if we are to one day solve the so-called *protein folding problem* and design new proteins with enhanced biophysical properties, one ought to be aware of the conformational changes involved in protein folding.

Application of structural techniques to monitor protein folding *in vivo* is hardly feasible. Nowadays, the most common experimental strategies involve protein denaturation *in vitro*, in conjunction or not with renaturation methodologies. These approaches and the limits of their applicability will be addressed in the following subsections.

C.1. *Insights into Protein Folding from Protein Unfolding* in vitro

At first sight, transfer of data from protein unfolding experiments and computer simulations to folding processes *in vivo* is a challenging and risky task. Nevertheless, it has been demonstrated that accurate

information may derive from such indirect approach to protein folding. Firstly, the transition state is known to be the same for folding and unfolding reactions of a two-state folder protein. Thus, the transition state for folding can be defined by measuring or theoretically simulating unfolding reaction kinetics (Fersht & Daggett, 2002). In addition, integrated applications of protein engineering and chemically induced denaturation have proved to be very useful for interpreting the structure of, not only transition states (Best, *et al.*, 2002; Fersht & Sato, 2004), but also other intermediary species (Best, *et al.*, 2002; Campos, *et al.*, 2004) along the protein folding pathway. Intermediary species with native-like features have been suggested to facilitate the search for the native conformational state (Creighton, *et al.*, 1996; Roder & Colón, 1997).

Finally, an emerging view has been emphasizing the importance of a thorough characterization of residual structure in unfolded states for clarification of early events in protein folding, because it has been proposed to correspond to initiation points of the folding pathway (Wong, *et al.*, 1996). Nuclear magnetic resonance has been considered the best method to map denatured states (Chatterjee, *et al.*, 2005; Yi, *et al.*, 2000).

C.2. *Simulation of Protein Folding by Protein Refolding* in vitro

In recent years, knowledge on the nature of protein folding processes has had important contributions from experimental data obtained through denaturation-renaturation strategies. In other words, induction of an order-to-disorder transition is followed by re-establishment of native (or *quasi*-native) conditions, and the ensuing conformational events undergone by the protein are monitored. On restoration of native conditions, a protein may or may not recover its original conformation. Possible causes for denaturation irreversibility had already been discussed in a foregoing section. In case the protein succeeds in reattaining its native conformational state and reacquiring its biological activity, the process is designated *refolding* (Lesk, 2004).

In general, the rate of refolding is dependent neither on conditions used to destabilize the natively folded state, nor on the final conformational state of the denaturation transition. Rather, it strongly depends on the protein covalent structure and on the final refolding conditions. On average, proteins have been found to refold on the second to minute time-scale (Creighton, 1988). Folding of some subpopulations within the starting denatured-state ensemble can be delayed by intrinsically slow events like, for example, *cis-trans* peptide bond isomerization (Creighton, 1988; Creighton, *et al.*, 1996; Reimer, *et al.*, 1998). And, many different factors may influence the final yield of *in vitro* refolding by driving the process to a kinetic partitioning into productive and non-productive events (Mendoza, *et al.*, 1991; Sadana & Vo-Dinh, 2001). The latter could consist of misfolding and / or aggregation.

In vivo and *in vitro* folding mechanisms of small proteins up to 10,000 – 15,000 Da (which is approximately the size of a domain of a large protein) are similar. Thus, it has been taken for granted that knowledge obtained from studying mechanisms of folding of small, fast-folding proteins *in vitro* can be confidently applied to their folding *in vivo*, as well as to the folding of individual domains in larger proteins (Fersht & Daggett 2002). *In vitro* protein refolding has allowed reaching conclusions on folding mechanisms,

as well as on formation of intermediates and on boundaries of the folding core. A telling example is that of a study performed by Campana and collaborators (2001) on refolding of frutalin, a tetrameric lectin. Their data have, not only hinted at a nucleation-condensation mechanism for frutalin folding, but also allowed the detection of an intermediary species and identification of a sugar-binding site as the folding core of the protein (Campana, *et al.*, 2001).

D. *Protein Stability*

By disrupting a protein structure, one can study, not only its folding pathway(s) and architecture, but also its energetics. This impels the introduction of a new subject: *protein stability*. From an inclusive viewpoint, stability might be defined as the extent to which a protein is able to maintain its chemical and conformational integrity and native activity, when submitted to perturbation by physical and / or chemical factors. However, the term 'stability' has been adapted to a multitude of contexts in protein science, so that different expressions have appeared.

The concept lying beneath the phrase '*conformational stability*' results from a constriction of the wide-ranging definition of stability to the conformational robustness of a protein folded state. The use of this expression is therefore restricted to situations in which no covalent changes occur (Pace, *et al.*, 1990b; Pfeil, 1998). Whenever modifications, often irreversible, in the covalent structure of a protein take place, another aspect of protein stability is called into play, the *chemical stability* (Pace, *et al.*, 1990b).

At most times, the concept of conformational stability claims a quantitative evaluation in energetic terms. However, throughout the current literary review and for the remainder of this dissertation, the expression 'conformational stability' will be employed in a qualitative sense. The difference between the Gibbs free energies of folded and unfolded states of a protein is more accurately coined *thermodynamic conformational stability* (Pfeil, 1998).

D.1. *Marginal Conformational Stability of Folded States*

Maintenance of the native state of a protein is generally restricted to specific environmental conditions, particularly, within narrow ranges of pH and temperature values, or concentrations of other compounds (Jaenicke, 1991). Subtly overstep these boundaries and proteins lose their ordered structure and functional efficiency. In fact, one of the most peculiar hallmarks of proteins is the marginal thermodynamic conformational stability of the folded state of proteins with respect to their unfolded state (under native conditions) (Doster & Friedrich, 2005). The free energy of stabilization of globular proteins in solution is tantamount to only a few weak intermolecular interactions (Jaenicke, 2000). Correspondingly, thermodynamic conformational stability values computed for globular and monomeric proteins under near-physiological conditions range between +6 and +14 kcal/mol. The positive sign reflects the energetic

favourableness of the folded state *vis-à*-vis the unfolded state (Pace, *et al.*, 2005; Pfeil, 1981); and the more positive the thermodynamic conformational stability value, the more stable the protein is (Betz, 1993).

D.1.1. *Physical and Molecular Foundations*

Inadequate stability and its consequences may restrain industrial, biotechnological and medical applications of proteins. Long-term stability and absence of aggregation under harsh conditions are often required. Sought of processes meant at positive regulation of protein stability, or *stabilization*, has become a challenging task. In search of clear-cut stabilization strategies, many principles, factors and mechanisms responsible for the folding and conformational stability of protein folded states have been unravelled. Even though they are still far from being completely understood, it is well-established that the natively active conformational state of a protein is a consequence of an intricate and fragile equilibrium between destabilizing and stabilizing forces and features (Vanhove, *et al.*, 1995). And, different proteins appeal to different combinations of interactions and other intrinsic attributes in order to assure preference for the natively folded state over denatured states under physiological conditions, and to defy inappropriate environmental conditions (Jaenicke, 2000).

The numerous, albeit weak, non-covalent interactions that protein atoms engage, either with solvent or with other protein atoms, in any conformational state, contribute as an interdependent whole to the subtle energetic difference between folded and unfolded states (Genzor, *et al.*, 1996; Neet & Timm, 1994). In this respect, optimization of the following types of forces is recognized to positively impact protein conformational stability: (i) van der Waals interactions in densely packed hydrophobic cores (Braxton, 1996; Chen, *et al.*, 2000; Guerois, *et al.*, 2005; Sneddon & Tobias, 1992); (ii) non-local charge-charge interactions (Vieille & Zeikus, 2001); (iii) cooperative networks of salt bridges and ion pairs in general at protein surface (Nakamura, 1996; Zhou, 2002); (iv) aromatic-aromatic interactions (Burley & Petsko, 1985; Serrano, *et al.*, 1991; Vieille & Zeikus, 2001); (v) intramolecular cation-π interactions involving aromatic groups (Dougherty, 1996; Vieille & Zeikus, 2001); (vi) intramolecular charge-dipole interactions (Guerois, *et al.*, 2005; Nakamura, 1996); (vii) intramolecular dipole-dipole contacts between polar groups not directly hydrogen-bonded to each other (Lockhart & Kim, 1993); and (viii) intramolecular hydrogen bonds, in particular, neutral-charged hydrogen bonds (Myers & Pace, 1996; Pace, 2001; Pace, *et al.*, 1996; Tanner, *et al.*, 1996; Vanhove, *et al.*, 1995).

Other stabilizing strategies consist of, for example: (i) oligomerization (Neet & Timm, 1994; Thoma, *et al.*, 2000) (ii) loop shortening and tying down (Russell, *et al.*, 1997; Schimmele & Plückthun, 2005); (iii) abundance of proline residues – the amino acid exhibiting the lowest conformational entropy (Jaenicke, 2000); and (iv) glycosylation (Solá & Griebenow, 2006). Aside from intrinsic structural determinants, protein conformational stability may also be incremented by water molecules (consult the following subchapter), salts, cofactors and substrates (Braxton, 1996; Takai, *et al.*, 1997).

With regard to cellular life in particular, other specific mechanisms have been developed in order to keep proteins in their natively folded state and to enhance their intrinsic stability. Such *in vivo* stabilization

mechanisms may involve molecular chaperones, protein repair enzymes and high concentrations of compatible solutes (Jaenicke, 2000; Sterner & Liebl, 2001).

D.1.2. *Biological Advantages*

From the biological standpoint, marginal conformational stability of proteins affords several gains, namely: (i) control of protein levels in the cell, for low conformational stability will facilitate degradation at specific rates (Makhatadze, 2005; Schimmele & Plückthun, 2005); (ii) transport of proteins across the membrane among diverse compartments in the cell, and other cellular processes involving folding-unfolding transitions (Matouschek, 2003); (iii) regulation of folding mechanisms, preventing accumulation of aberrant intermediary species such as aggregates (Doig & Williams, 1992); (iv) ready responses to environmental changes (Lazaridis & Karplus, 2003); and (v) balance between a well-defined and adequately rigid three-dimensional structure, required for functional specificity, and a tolerable flexibility, essential for conformational changes involved in ligand binding, catalysis, and allosteric regulation (Bettati, *et al.*, 2000; Jaenicke 2000; Szeltner & Polgár, 1996; Závodszky, *et al.*, 1998).

2. *PROTEIN HYDRATION*

The primary, secondary, tertiary and quaternary structures cannot fully account for all observable properties of a protein. Surrounding environments unarguably influence protein folding, conformation, dynamics, stability and function. *In vivo*, proteins essentially exist either in aqueous environments such as the solvent within the cell, or in non-aqueous environments such as membranes (van Holde, *et al.*, 1998). The intricate relationship between a protein and its surrounding environment is the subject to be covered here. Due to the vastness of this topic, the main goals of this subchapter are to succinctly review structural and dynamical aspects of hydration and to highlight its influence on protein conformational stability.

A. *Structural and Dynamical Characteristics*

The general solution process is referred to as *solvation*. *Hydration* is a term specifically used to designate solvation by water (Philips & Pettitt, 1995). The great majority of water molecules are bound by at least one hydrogen bond to proton acceptors like oxygen atoms, or donors like nitrogen centers, but with a preference for the former (Baker, 1995). Thus, water is mainly a proton donor. About half the hydrogen bonds are made with the α-carbonyl groups of the polypeptide backbone. The remaining hydrogen bonds are formed with α-amide groups and with polar atoms of side chains (Daune, 1999). Besides hydrogen bonds, it

is of relevance to mention the occurrence of electrostatic (Koenig, *et al.*, 1975) and van der Waals interactions between protein groups and water molecules (Pace, 2001).

A.1. *Surface Hydration*

A micellar model used to be considered when describing a folded protein molecule in an aqueous solution. According to this model, hydrophilic amino acid residues and polar heads of amphiphilic residues tend to seek the exterior, where they can be solvated through contact with the water (Daune, 1999). Conversely, exposure of hydrophobic moieties to solvent is minimized, as interactions between them and water are generally unfavourable. The tendency for burying and clustering hydrophobic side chains in order to sequester themselves in the interior of proteins, away from the contact with water, is designated *hydrophobic effect* (Spolar, *et al.*, 1989). The hydrophobic effect is widely asserted as one of the major promoters of folding of globular proteins (Chan & Dill, 1990; Pace, *et al.*, 1996). Nonetheless, it has been observed that hydrophobic residues are not strictly placed in the interior and the hydrophilic ones rigorously placed at the protein surface (Daune, 1999). In fact, protein surface is composed of hydrophobic and hydrophilic regions with comparable dimensions (Lins, *et al.*, 2003).

In an aqueous solution, the surface of a soluble, globular protein is surrounded by several layers of water molecules non-covalently bound to the protein by hydrogen bonds – the *hydration shell* (or the *water shell*), with average residence times in the sub-nanosecond range (Denisov & Halle, 1996; Khmelnitsky, *et al.*, 1991a; Khmelnitsky, *et al.*, 1991b; Otting, *et al.*, 1991). The set of ordered waters directly bound to electrically neutral or charged polar groups defines the *first hydration shell* (or the *first water shell*) (Eisenhaber, 1996). Such water layer is surmised to be an integral part of the protein (Eisenhaber, 1996; Pethig, 1995). Binding to the protein surface is stronger for the water molecules of the first hydration shell. The strength of binding will decrease in subsequent layers (Whitaker, 1994). Chalikian and collaborators (1996) proposed that hydration shells of polar-neutral groups in proteins are particularly enhanced, for these groups favour development of water networks which incorporate water molecules from the second and, possibly, even from the third coordination spheres. In these networks, it has been noted that a water molecule from the first hydration shell may simultaneously establish hydrogen bonds with two or more polar groups at the protein surface. On becoming highly immobilized, these water molecules behave similarly to protein polar groups, thereby facilitating formation of hydrogen bonds with water molecules from subsequent hydration layers (Chalikian, *et al.*, 1996).

The water shell around a protein molecule cannot be considered a rigid entity. Its constituent water molecules are in equilibrium with *bulk water*. Nonetheless, they are structurally, dynamically and thermodynamically altered by chemical and topographical properties of protein surface (Merzel & Smith, 2002). In reality, only at relatively large distances from the protein surface are the characteristics of bulk water conserved (Eisenhaber, 1996). For instance, the hydration shell may have locally higher density as

compared to bulk water, owing to electrostriction around charged groups. According to Beck and collaborators (2003), the first hydration shell of negatively charged groups exhibits higher density and longer residence times relative to the first water layer of the majority of other groups. On the other hand, packing around hydrophobic groups leads to lower water density as compared to the bulk solvent (Beck, *et al.*, 2003; Svergun, *et al.*, 1998).

Protein hydrophobic groups are unable to establish hydrogen bonds with water molecules (Spolar, *et al.*, 1989) and to orient local waters (Beck, *et al.*, 2003). And, thus, hydrophobic moieties at the protein-water interface are merely surrounded by clusters of '*icebergs*', which consist of transient, hydrogen-bonded, ordered arrays of water molecules (Darby & Creighton, 1993; Finney, *et al.*, 1993). In 'icebergs', water molecules are organized into hydrogen-bonded polygons, with pentagons occurring more frequently than hexagons (Head-Gordon, 1995; Pertsemlidis, *et al.*, 1996); whereas a tetrahedral hydrogen-bond geometry is, in principle, retained by hydration water molecules on the remaining protein surface (Nakasako, 2002).

A.2. *Buried Water Molecules*

Apart from external water, proteins display ordered water molecules lodged in small cavities (Ernst, *et al.*, 1995; Hubbard, *et al.*, 1994; Takano, *et al.*, 2003). A water molecule within a protein that cannot be connected by a continuous series of water-water hydrogen bonds to bulk water molecules is classified as a *buried* or *internal water molecule* (Williams, *et al.*, 1994).

Some cavities accommodate only a single water molecule; others are large enough to contain small clusters. *Discrete buried water molecules* engage at least a couple of hydrogen bonds with protein atoms (more commonly, three or four) (Halle, *et al.*, 2005). C*lusters of buried water molecules* often form chains, in which each water molecule is hydrogen-bonded to the next, but it is also hydrogen-bonded to protein atoms (Williams, *et al.*, 1994).

In spite of being apparently isolated, there is an exchange of buried water molecules with bulk solvent, presumably through a general breathing motion of the protein and narrow channels that link these internal cavities to the protein surface (Baker, 1995). Obviously, it is much slower than the exchange process between hydrogen-bonded water at the protein surface and the bulk solvent (Otting, *et al.*, 1991).

Burial of a water molecule within many of the internal cavities of a protein may be thermodynamically unfavourable if they lack enough hydrogen bonds to compensate for the loss of bonds formed with bulk water. Moreover, in case a cavity is small, there will be a higher probability for van der Waals repulsion between water and the protein (Williams, *et al.*, 1994). On the other hand, favourable intermolecular interactions in bulk water may be compensated by a large cavity occupied by an internal hydrogen-bonded water cluster, or a hydrated cavity with a large electric field, produced by ionized side chains in close proximity (Halle, *et al.*, 2005).

Internal water molecules are often conserved through evolution and within families of homologous proteins, for instance, serine proteinases (Sreenivasan & Axelsen, 1992). Subsequently, the extent of hydration of cavities has been presumed to exert a noteworthy influence on the structure and activity of a

protein molecule (Halle, *et al.*, 2005).

B. *Contributions to Protein Folding and Conformational Stability*

The slightly bad-solvent character of water might well be the way nature found to cause spontaneous folding of proteins (Schellman, 2003). In fact, water plays a vital role in the establishment of non-covalent intramolecular interactions accountable for unique three-dimensional structures of proteins. An enlightening example of this influence is the hydrophobic effect (Spolar, *et al.*, 1989) which, in turn, induces the need for satisfaction of hydrogen-bonding potential of buried polar groups (McDonald & Thornton, 1994; Pace, *et al.*, 1996; Stickle, *et al.*, 1992). Furthermore, water frequently acts as a direct mediator in a variety of intramolecular interactions. Water molecules in general are suitable for bridging between protein atoms, due to their small size and double-donor / double-acceptor hydrogen-bonding capacity. As such, water molecules can make great contributions to protein conformational stability: (i) mediating hydrogen bonds between partners that might be of the same type (both acceptors and both donors) and, hence, cannot form hydrogen bonds directly (Baker, 1995; Williams, *et al.*, 1994; Xu, *et al.*, 1997); and (ii) bridging between protein atoms otherwise unable to interact owing to steric or geometric constraints (Baker, 1995; Xu, *et al.*, 1997).

Regarding to buried water molecules in particular, evidence points to meaningful contributions from filling intramolecular cavities with solvent molecules to protein conformational stability. Indeed, whenever a cavity is adequately large and displays sufficient hydrogen-bonding groups in a suitable orientation, its hydration might increase protein conformational stability by: (i) hydrogen-bonding to otherwise unsatisfied protein hydrogen-bonding groups; and (ii) supplying otherwise missing favourable van der Waals interactions for atoms bordering the cavity (Williams, *et al.*, 1994). 'Charge spreading' is an additional role attributed to internal water molecules. Circumstances can occur in which charged side chains in the protein interior are not compensated. Likewise isolated hydrogen bond donors and acceptors, unpaired buried charges are energetically too expensive to exist in the hydrophobic core(s) (Nakamura, 1996). In order to counterbalance such unpaired charges, buried water molecules can either (Baker, 1995): (i) link charged internal side chains that cannot make a direct contact; (ii) help create polar microenvironments; or (iii) become protonated (H_3O^+) and bind a non-neutral group in case it is negatively charged.

3. *PEPSIN A: AN ARCHETYPAL ASPARTIC PROTEINASE*

Cathepsin D, β-secretase and HIV-1 proteinase have been implicated in mechanisms underlying human pathologies which have received most attention in modern societies. These proteins act as *hydrolases*, and are included in one of the major sub-subclasses of *proteolytic enzymes* recognized by the Enzyme Commission, the so-called *aspartic proteinases* (EC 3.4.23). This group of proteinases includes one of the first enzymes to be discovered (Schwann, 1836 as quoted by Fruton, 2002) and purified in crystalline form

(Northrop, 1930a; Northrop, 1930b; Northrop, 1933) – *pepsin A* (EC 3.4.23.1), which is by far the most extensively studied aspartic proteinase.

In what follows, a brief review of diversity, general characteristics and importance of aspartic proteinases will be presented. Full coverage of the vast amount of knowledge about aspartic proteinases is beyond the scope of this dissertation. A major focus of this subchapter will be porcine pepsin A. Its specific structural features and catalytic properties, together with pepsinogen maturation, will be emphasized.

A. *Aspartic Proteinases*

A.1. *Classification and Distribution*

A hierarchical, structure-based classification of aspartic proteinases was recently released by Rawlings and co-workers (2004), and it is available at the *MEROPS* database (http://merops.sanger.ac.uk/). On account of similarities in primary structure, aspartic proteinases were sorted into 14 different *families*. Homologous families were gathered in a *clan*, on the basis of comparisons between tertiary structures or sequences around active site residues. Six different clans were distinguished within this group of proteolytic enzymes.

Aspartic proteinases are widely spread in Nature. Table 1.1 includes examples of aspartic proteinases isolated from different sources as well as corresponding families and useful references.

Table 1.1 – Examples of aspartic proteinases from different sources.

Source	Examples of Aspartic Proteinases	Family*
Animals	Pepsin A[1]	A1
	Gastricsin[2]	A1
	Chymosin[3]	A1
	Renin[4]	A1
	Cathepsin D[5]	A1
	β - Secretase[6, 7]	A1
Plants	Cardosin A[8]	A1
	Nepenthesin[9]	A1
Fungi	Rhizopuspepsin[10]	A1
	Penicillopepsin[11]	A1
	Endothiapepsin[12]	A1
Protozoa	Plasmepsin[13]	A1
Viruses	HIV-1 Proteinase[14, 15]	A2
	Cauliflower Mosaic Virus - Type Peptidase[16]	A3
Bacteria	Omptin[17]	A26
Archaea	Thermopsin[18]	A5

Notes: (*) Classification adopted by the *MEROPS* database (Rawlings, *et al.*, 2004).
References: (1) Fruton, 2002; **(2)** Martin, *et al.*, 1982; **(3)** Gilliland, *et al.*, 1991; **(4)** Inagami, 1989; **(5)** Pain, *et al.*, 1985a; **(6)** Haniu, *et al.*, 2000; **(7)** Hong, *et al.*, 2000; **(8)** Frazão, *et al.*, 1999; **(9)** Athauda, *et al.*, 2004; **(10)** Suguna, *et al.*, 1987; **(11)** Hsu, *et al.*, 1977; **(12)** Blundell, *et al.*, 1990a; **(13)** Prade, *et al.*, 2005; **(14)** Blundell, *et al.*, 1990b; **(15)** Kovalskyy, *et al.*, 2005; **(16)** Torruella, *et al.*, 1989; **(17)** Vandeputte-Rutten, *et al.*, 2001; **(18)** Fusek, *et al.*, 1990.

A.2. *General Insights into Molecular and Catalytic Properties*

A substantial degree of similarity is shared by aspartic proteinases, especially, in regard to, secondary structural motifs, molecular shape and catalytic machinery (Davies, 1990; Tang & Wong, 1987). Nonetheless, considerable differences have also been reported within this group of enzymes, particularly, in terms of catalytic properties, localizations and, thus, biological functions (Koelsch, *et al.*, 1994). Such diversity, in conjunction with a growing body of information concerning sequence and structure and a progressive enlargement of the list of members of this sub-subclass of proteolytic enzymes, has rendered hard and dangerous any attempt to withdraw generalizations with respect to aspartic proteinases. In view of this, the ongoing subsection shall concentrate on characteristics commonly displayed by aspartic proteinases belonging to family A1, whose type proteinase is pepsin A. According to the *MEROPS* database, this is the largest family of aspartic proteinases, and it is deemed the type family of clan AA. Apart from pepsin A, family A1 comprises other gastric endoproteinases like, for example, gastricsin or chymosin, together with the lysosomal proteinase – cathepsin D, or the renal proteinase – renin, among others (see Table 1.1). Clan

AA further embraces families A2, A3, A9 and A11, whose representatives are HIV-1 proteinase, cauliflower mosaic virus - type peptidase, spumapepsin and copia transposon peptidase, respectively (Rawlings, *et al.*, 2004).

A.2.1. *Zymogens and Mechanisms of their Activation*

In contrast to retroviral counterparts, eukaryotic aspartic proteinases are biosynthesized as inactive proenzymes, *i. e. zymogens*. In zymogens, an N-terminal extension of the mature enzyme, the so-called *prosegment* (or *prosequence*), interacts in nonsubstrate-like manners with preformed, active conformations of the catalytic apparatus. Consequently, the active site generally becomes sterically inaccessible and, thus, binding of substrates is hampered (Khan & James, 1998; Khan, *et al.*, 1999; Sielecki, *et al.*, 1991). Prosegments of aspartic proteinases from family A1 are generally composed of 44 – 50 amino acid residues, and appear to be homologous (Foltmann, 1988; Foltmann & Pedersen, 1977; Khan, *et al.*, 1999; Tang & Wong, 1987).

Irreversible conversion of pepsinogen (for further details, *vide* subsection 3.B.1. '*Conversion of Pepsinogen A into Pepsin A*') and other gastric zymogens (James & Sielecki, 1986; Kageyama, 2002; Richter, *et al.*, 1998), seminal protease zymogen (Ruenwongsa & Chulavatnatol, 1975), or procathepsin D (Wittlin, *et al.*, 1999) into active enzymes is triggered by a drop in pH. Initially, the first 60 amino acid residues of the precursor undergo a gross conformational rearrangement. Subsequently, the prosegment is proteolytically removed, thereby exposing the catalytic apparatus (James & Sielecki, 1986; Koelsch, *et al.*, 1994; Tang & Wong, 1987). On the basis of the extent of dependence on molecular assistance, three different types of mechanisms of zymogen processing have been distinguished: (i) complete self-processing, which has been reported for pepsinogen activation (Richter, *et al.*, 1998; Kageyama, 2002); (ii) self-processing partially assisted by a different proteinase, which has been detected during maturation of zymogens of lysosomal proteinases (Conner, 1989; Samarel, *et al.*, 1989; Wittlin, *et al.*, 1999); and (iii) fully assisted processing, which is known to occur during conversion of prorenin to renin (Shinagawa, *et al.*, 1990).

The traditional view of the role of the prosegments of aspartic proteinases solely as inhibitors of the active site is changing, and they have also been deemed decisive in proper folding (Foltmann, 1988), and possibly in interacting with cellular receptors for targeting (Koelsch, *et al.*, 1994).

With only a few exceptions, precursors of plant aspartic proteinases contain an extra segment comprising approximately 100 amino acid residues, the so-called *plant-specific insert* (PSI). It is located within the C-terminal domain of the corresponding mature enzyme. It may be totally or partially excised or retained during generation of active proteinase from inactive zymogen. The role of PSI is still not elucidated, but it has been correlated with the delivery of the aspartic proteinase to the vacuole (Mutlu & Gal, 1999; Simões & Faro, 2004).

A.2.2. *Three-Dimensional Structure*

The overall tertiary structure of eukaryotic aspartic proteinases consists of two homologous globular domains, whose secondary structure is dominated by pleated sheets with relatively few α-helices (Blundell, *et al.*, 1990a; Cooper, *et al.*, 1990; Davies, 1990; Tang, *et al.*, 1978). In the *Structural Classification of Proteins* (*SCOP*) database, aspartic proteinases are included in the all-β-protein class (Murzin, *et al.*, 1995). In general, the two domains of aspartic proteinases and their active site residues are related by an intramolecular, approximate two-fold symmetry axis (Cooper, *et al.*, 1990; Fruton, 2002; Tang, *et al.*, 1978). Intradomain two-fold symmetry has also been detected, albeit it is less obvious (Andreeva, *et al.*, 1984; Blundell, *et al.*, 1979; Blundell, *et al.*, 1990a; Cooper, *et al.*, 1990). Interdomain interfaces of pepsin-like enzymes include a continuous chain of hydrogen-bonded groups. A fundamental piece of the system of interdomain interactions is the so-called '*fireman's grip*' – a conserved arrangement of hydrogen bonds involving main and side chain groups of residues near the interdomain catalytic dyad which is presumed to stabilize the geometry of the active site (Andreeva & Gurskaia, 2006; Blundell, *et al.*, 1990a; Strisovsky, *et al.*, 2000).

Seventeen water molecules were found to be conserved within a set of crystal structures of eight native aspartic proteinases. Different roles have been ascribed to these solvent molecules, namely: (i) overall conformational stabilization; (ii) maintenance of the geometry of the active site and fixation of the conformation of vicinal regions required for enzymatic function; and (iii) direct involvement in the mechanism of peptide hydrolysis (Prasad & Suguna, 2002).

A.2.2.1. *Catalytic Apparatus*

The catalytic apparatus of pepsin-type enzymes is located at the interface between the two domains, and each of them contributes with one of the pair of aspartic residues that is responsible for activity (Asp32 and Asp215) (Andreeva & Rumsh, 2001; Cooper, *et al.*, 1990). Catalytic aspartic residues are related by the interdomain dyad. Their side chains are held coplanarly and involved in an intricate hydrogen-bonded network with neighbouring residues (Andreeva & Rumsh, 2001; Blundell, *et al.*, 1990a), which imparts rigidity to the catalytic apparatus (Davies, 1990; Lin, *et al.*, 1992b). The substrate-binding cleft extends nearly the entire width of the enzyme molecule, running across the junction of the two domains, and it can accommodate at least 7 residues of a substrate or inhibitor (Dunn, *et al.*, 1995; Kay & Dunn, 1992; Sampath-Kumar & Fruton, 1974). A flexible loop, which is commonly known as the '*flap*', projects over the cleft forming a channel into which the substrate binds and shields the active site from the solvent region (Blundell, *et al.*, 1990a; Okoniewska, *et al.*, 1999). Substrates bind to the active site and 'flap' mainly through an extensive network of hydrogen bonds (Andreeva, *et al.*, 1995; Dunn & Hung, 2000).

A.2.2.2. *Evolution*

A large number of eukaryotic aspartic proteinases are two-domain monomeric molecules, while retroviral proteinases are only functional as dimers of identical polypeptides (Jaskolski, *et al.*, 1990; Pearl & Taylor, 1987). Even so, eukaryotic and retroviral aspartic proteinases have been considered to have some degree of structural equivalence. Each subunit of aspartic proteinases of retroviral origins bears close resemblance to a domain of the pepsin molecule, and contributes with one catalytic aspartic residue as well (Jaskolski, *et al.*, 1990; Pearl & Taylor, 1987; Rao, *et al.*, 1991). Comparisons between the interdomain interface of two-domain, monomeric pepsin-type aspartic proteinases and the intersubunit interface of dimeric retroviral aspartic proteinases further reveal structural homology (Rao, *et al.*, 1991). These observations have lent support to the proposal for an evolutionary relationship between retroviral aspartic proteinase genes and the early ancestor of two-domain monomeric proteinases. It has been suggested that the gene encoding the present-day two-domain monomeric proteinase derived in evolution from an ancestor encoding a smaller dimeric proteinase by gene duplication and fusion. The latter would be itself the evolutionary product of duplication, followed by fusion, of a gene encoding an ancestral monomeric protein (Lapatto, *et al.*, 1989; Lin, *et al.*, 1992a; Miller, *et al.*, 1989; Tang, *et al.*, 1978).

A.2.3. *Catalysis*

As briefly aforementioned, catalytic activity of aspartic proteinases is directly dependent on two aspartic residues. Surroundings of the catalytically active aspartic residues are highly conservative, for consensus sequences (Asp – Thr – Gly or Asp – Ser – Gly) in both N- and C-terminal domains have been detected in proteinases from clan AA (Rawlings & Barrett, 1995). Aspartic proteinases generally function in acidic conditions and, thus, their occurrence is limited to some specific locations.

All of the known members of clan AA are endoproteinases (Rawlings & Barrett, 1995). The majority of the members of family A1 show preference for cleaving bonds in extended peptide substrates with hydrophobic residues occupying P_1 and P_1' positions (Dunn, *et al.*, 1986; Dunn, *et al.*, 1987; Fruton, 1970). However, specificities within the group of aspartic proteinases are diverse, insomuch as they range from a highly strict requirement for renin (Dhanaraj, *et al.*, 1992) to broad preferences of pepsin (Fruton, 1970) and fungal enzymes (Fusek, *et al.*, 1995). Substrate specificity is determined by several hydrophobic residues nearby the catalytic aspartic residues and by residues on the 'flap' (Rawlings & Barrett, 1995). Functional selection of specificity was achieved by discrete modifications in the large substrate-binding cleft, in terms of size of residues in the specificity pockets and position of secondary structural elements forming the pockets (Cooper, 2002; Dhanaraj, *et al.*, 1992).

Members of clan AA are all reversibly inhibited by pepstatin A, which acts as a transition-state analogue inhibitor (Marciniszyn, *et al.*, 1977). Diazoacetylnorleucine methyl ester in the presence of cupric ions (Rajagopalan, *et al.*, 1966; Takahashi, *et al.*, 1972) and 1, 2-epoxy-3-(*p*-nitrophenoxy) propane (Chen & Tang, 1972; Tang, 1971) are two covalently reacting inhibitors of pepsin, which have been used as diagnostic

reagents for aspartic proteinases.

A.2.3.1. *Mechanism for Peptide Hydrolysis*

Taking into account that aspartic proteinases bear similarities in their three-dimensional structures and catalytic machinery, they are believed to operate a common catalytic mechanism for proteolytic cleavage (Dunn, *et al.*, 1986; Kay & Dunn, 1992). Nowadays, most researchers agree that catalysis of peptide hydrolysis by aspartic proteinases proceeds by a general-acid / general-base mechanism, additionally involving a nucleophilic attack (Park, *et al.*, 2000).

In pepsin-like enzymes, one of the catalytic aspartic residues is charged and acts as a general base (Asp215); while the other one is protonated on the outer oxygen atom of its carboxyl group and acts as a general acid (Asp32) (Topol, *et al.*, 1995). Residues adjacent to the catalytic apparatus play a role in conserving the charged state of Asp215 and the protonated state of Asp32 (Andreeva & Rumsh, 2001). A water molecule bridges the side chains of the active aspartic residues. It is tightly bound to the active site through a bifurcated hydrogen bond formed with the inner oxygen atoms of the side chains of the catalytic aspartic residues. The water oxygen atom and the proton of the neutral aspartic residue form another hydrogen bond. Hydrogen bonds orientate the water molecule diagonally across the active site (Andreeva & Rumsh, 2001; Beveridge & Heywood, 1993; Topol, *et al.*, 1995; Veerapandian, *et al.*, 1992).

In a first step of the catalytic mechanism (see Figure 1.1), the water molecule nucleophilically attacks the carbonyl carbon atom of the scissile bond. Concurrently, the ionization of the active site undergoes a reversal: a carboxylic proton is transferred from the Asp32 to the carbonyl oxygen atom of the substrate, and another proton is transferred from the attacking water molecule to the outer oxygen atom of the carboxyl group of Asp215. The tetrahedral intermediate generated is stabilized by hydrogen bonds formed between a gem-diol unit and side chains of catalytic aspartic residues. Finally, the latter proton moves to the nitrogen atom of the scissile bond, triggering the decomposition of the bound complex in order to form products (Beveridge & Heywood, 1993; Veerapandian, *et al.*, 1992).

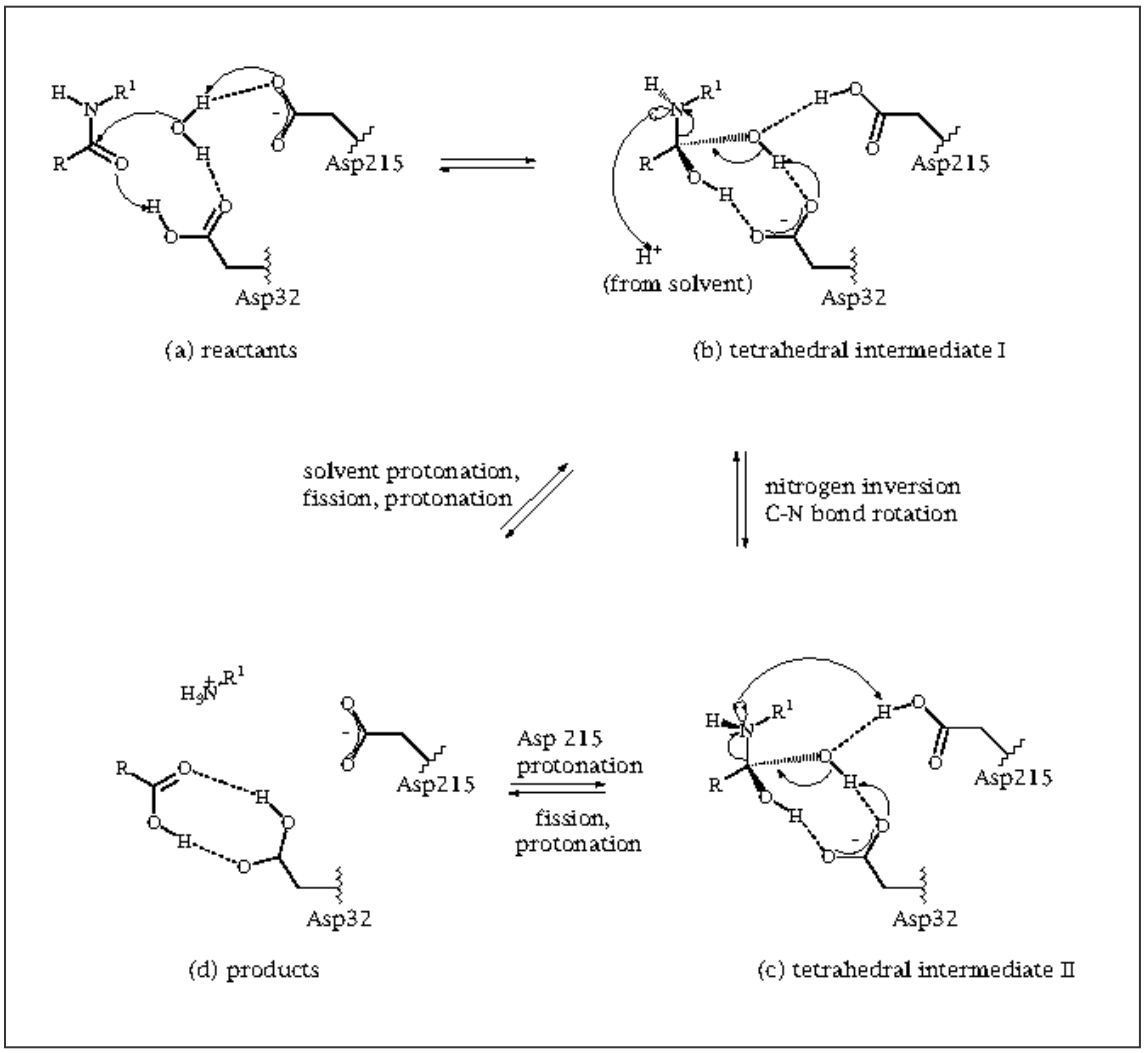

Figure 1.1 – The mechanism currently accepted for peptide hydrolysis by aspartic proteinases. ***In***: Veerapandian, *et al.*, 1992.

A.3. *Physiological, Pathological and Biotechnological Significance*

As illustrated in Table 1.2, aspartic proteinases have significant roles in a number of physiological and pathological mechanisms, and are involved in a few biotechnological processes.

Table 1.2 – Examples of biological roles and biotechnological applications of aspartic proteinases.

Aspartic Proteinases	Biological Roles / Biotechnological Applications
Renin	Blood pressure homeostasis[1]
HIV-1 Proteinase	Maturation of the HIV-1 particle[2]
β-Secretase	Amyloidogenic processing of amyloid precursor protein[3, 4]
Plasmepsin	Haemoglobin degradation in malaria[5]
Secreted Aspartic Proteinases from *Candida albicans*	Promotion of tissue invasion and damage in candidiasis[6]
Aspergillopepsin F	Hydrolysis of structural proteins in invasive aspergillosis[7]
Cathepsin D	Intracellular proteolysis[8] Metastasis of breast cancer[9]
Pepsin A	Hydrolysis of acid-denatured proteins in stomach[10] Mucosal injury in peptic ulcer disease and reflux oesophagitis[11, 12, 13] Depolymerization of chitosan[14] Solubilization of collagen[15]
Chymosin	Digestion of milk proteins in the stomach of fetal and neonatal mammals[16] Cheese manufacture[17]
Cardosin A	Hypothetical role in adhesion-mediated proteolytic mechanisms involved in pollen recognition and growth[18] Cheese manufacture[19]

References: **(1)** Haber, 1979; **(2)** Ross, *et al.*, 1991; **(3)** Vassar, 2001; **(4)** Hong, *et al.*, 2002; **(5)** Martins, *et al.*, 2003; **(6)** Naglik, *et al.,* 2003; **(7)** Lee & Kolattukudy, 1995; **(8)** Authier, *et al.*, 2002; **(9)** Liaudet-Coopman, *et al.*, 2005; **(10)** Fruton, 2002; **(11)** Cooper, 2002; **(12)** Pearson & Roberts, 2001; **(13)** Tang, *et al.*, 2005; **(14)** Kumar, *et al.*, 2007; **(15)** Bannister & Burns, 1972; **(16)** Foltmann, 1992; **(17)** Mohanty, *et al.*, 1999; **(18)** Faro, *et al.*, 1999; **(19)** Faro, *et al.*, 1992.

B. *Pepsin A*

Pepsin A has been deemed an archetype of the group of aspartic proteinases (Khan, 1999; Rawlings, *et al.*, 2004; Sielecki, *et al.*, 1990). It is the predominant proteolytic enzyme in the gastric juice of most vertebrates, starting the multi-step process of protein digestion. Pepsin A is able to split hydrophobic cores unfolded at low pH (Andreeva, 1994).

The best studied pepsins are those from the stomachs of high mammals, and most data available at open literature pertain to porcine pepsin A, which is the subject of the following subsections. A few striking differences in biophysical and catalytic properties have been detected on comparing mammalian and non-mammalian pepsins (Diaz-López, *et al.*, 1998; Pletschke, *et al.*, 1995).

In some vertebrates, pepsin A has shown to consist of several isoforms (Green, *et al.*, 1996; Meitner & Kassell, 1971; Tarasova, *et al.*, 1995). Correlations have been drawn between heterogeneity of pepsin A in certain species and feeding habits or types of food (Carginale, *et al.*, 2004; Kageyama, 2002). In respect of porcine pepsin A, mass spectroscopic data evidenced two variants with either an aspartic or tyrosine residue at the position 242 (Green, *et al.*, 1996).

B.1. *Conversion of Pepsinogen A into Pepsin A*

Pepsinogen A (*i. e.* the zymogen of pepsin A) is synthesized and stored in the chief cells of gastric mucosa (Elliott & Elliott, 2001). It comprises a single polypeptide chain of 370 amino acid residues (with a molecular mass of 40,000 Da), whose first 44 residues correspond to the prosegment (Sielecki, *et al.*, 1991). The large number of basic residues in the prosequence contributes to the conformational stability of pepsinogen A at neutral pH by neutralizing negative charges in the mature enzyme portion of the molecule. At the N-terminus, the prosegment adopts a β-strand conformation, which corresponds to the first strand of a six-stranded anti-parallel β-sheet at the back of the pepsinogen A molecule. The remainder of the prosegment consists of three short helical regions. And, these helices, together with the first 12 residues of the active enzyme moiety, cover and block the substrate-binding cleft (Hartsuck, *et al.*, 1992; Khan & James, 1998; Sielecki, *et al.*, 1991). Despite the displacement of an N-terminal fragment of the mature enzyme portion by the prosegment, the active enzyme moiety of pepsinogen A and pepsin A fold in an equivalent manner (see Figure 1.2).

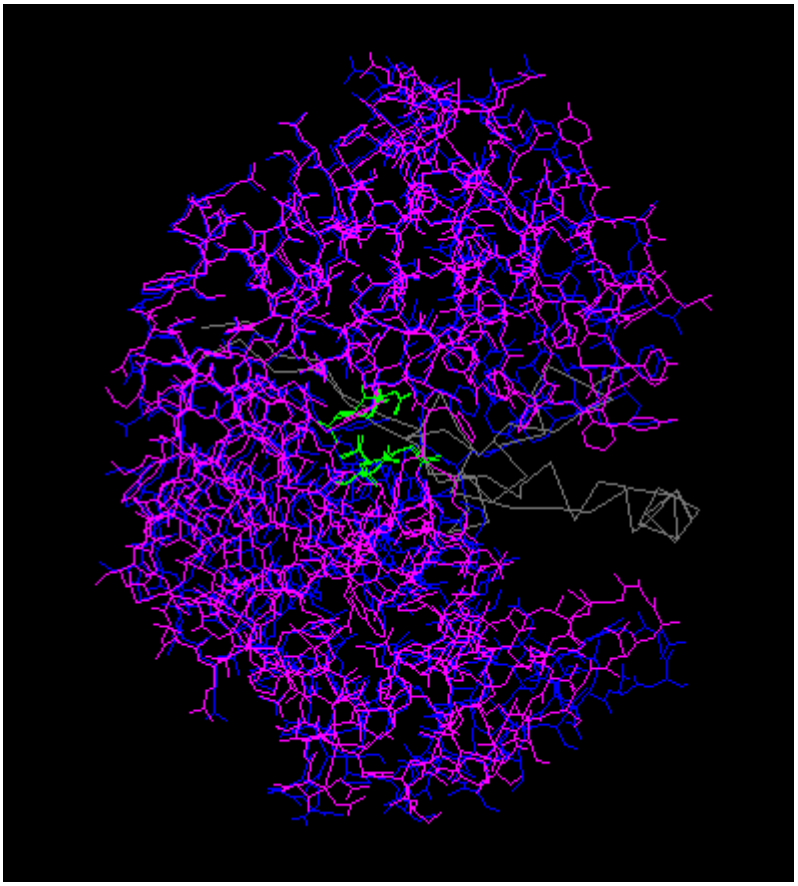

Figure 1.2 – Structural alignment of porcine pepsinogen A (PDB code *3psg*) and porcine pepsin A (PDB code *4pep*). As regards the zymogen, the active enzyme moiety (from Glu13 to Ala326) is shown in pink, and the prosegment and the first 12 residues of the active enzyme moiety are coloured in grey. Catalytic triads are shown in green. Porcine pepsin A (from Glu13 to Ala326) is coloured in blue. This figure was generated using the *Combinatorial Extension (CE) Method* (Shindyalov & Bourne, 1998).

Apart from electrostatic interactions between basic residues of the prosegment and acidic residues in the mature enzyme portion, hydrogen bonds and hydrophobic interactions contribute to binding of the prosegment to the active enzyme moiety (Richter, *et al.*, 1998). Specific interactions responsible for blockage of access to the S_1 and S_1' binding subsites and, hence, for inactivity of pepsinogen A have been identified. Tyr37p and Tyr9 establish two hydrogen bonds with the catalytic aspartic residues, Asp215 and Asp32, respectively (Richter, *et al.*, 1998; Sielecki, *et al.*, 1991). The negatively charged Asp215 is neutralized on forming an ion pair with Lys36p. An extensive hydrogen-bonding network in the active site of the zymogenic form presumably stabilizes the negative charge on Asp32 (Richter, *et al.*, 1999). Inactivation of the precursor of pepsin A is rationalized by the need to prevent damages in the host tissue and autoproteolysis (Khan & James, 1998).

In response to entrance of food in the stomach, the hormone gastrin produced by gastric endocrine cells is released into the blood, stimulating the secretion of pepsinogen A in the stomach (Elliott & Elliott, 2001; Koelsch, *et al.*, 1994). At pH values below 5, the acidic residues in the active enzyme portion become protonated and, thus, electrostatic interactions that hold the prosegment in place at neutral pH are disrupted.

Consequently, the active site is uncovered and the scissile bond of the prosequence is properly placed into the active site (Richter, *et al.*, 1998).

The next step in the maturation process consists of autocatalytic cleavage of the prosegment from the N-terminus of the zymogen (Richter, *et al.*, 1998), which is performed by the same active site that is responsible for the hydrolysis of substrates (Lin, *et al.*, 1989; Sinkovits, *et al.*, 2006). Proteolytic processing occurs by either a direct or sequential pathway. In the direct route, cleavage of the peptide bond Leu44p – Ile1 enables excision of the entire prosequence in one step. In the sequential route, the prosegment release occurs via a two-step process, whereby hydrolysis of one peptide bond within the prosegment (Leu16p – Ile17p) is followed by cleavage of the peptide bond connecting the prosequence to the active enzyme moiety. One- and two-step activation processes occur simultaneously (Kageyama & Takahashi, 1983). Furthermore, removal of the prosegment involves intra- and intermolecular reactions. In the former situation, the zymogen cleaves its own prosequence (Marciniszyn, *et al.*, 1976). In the latter situation, the prosequence is cleaved by a molecule of mature enzyme or an active intermediate. Activation occurs intramolecularly below pH 3, but the intermolecular autoproteolysis predominates at mildly acidic pH, *i. e.* between pH 4 and 5 (Al-Janabi, *et al.*, 1972; Richter, *et al.*, 1998). Intra- and intermolecular reactions yield equal active enzyme molecules (Varón, *et al.*, 2006).

Finally, the prosequence is dissociated from the active enzyme moiety and the residues at the N-terminus of pepsin A move approximately 40 Å to replace the N-terminus of the prosegment in the six-stranded anti-parallel β-sheet (Hartsuck, *et al.*, 1992).

In harmony with what was abovementioned, the role of prosequence in pepsinogen A is apparently not restricted to inhibition of the active site. Rather, it has been considered critical for correct folding. In fact, evidence from a study authored by Lin and collaborators (1992a) indicates that the N-terminal is unable to fold properly in the absence of the prosegment. On the other hand, the C-terminal domain is able to fold independently of this fragment of the zymogen (Lin, *et al.*, 1992a). In view of this, the prosegment has been proposed to have co-evolved with the N-terminal domain after an event of gene duplication (Koelsch, *et al.*, 1994).

B.2. *Structure: Specific Considerations*

Pepsin A is a monomeric protein consisting of 326 amino acid residues with a molecular mass of 34,530 Da (Sielecki, *et al.*, 1990). A striking feature of its amino acid composition is the predominance of acidic over basic residues (Tang, *et al.*, 1973). Pepsin A is a non-glycosylated protein; instead, there is a single phosphoryl group covalently linked to Ser68. The low pK_a for this group, together with the excess of acidic residues and unusual microenvironments of several of their side chain groups, contributes to a low isoelectric point (lower than 1) and, hence, ensures a net negative charge of pepsin A in strongly acidic media (Andreeva, *et al.*, 1984; Sielecki, *et al.*, 1990).

General features previously mentioned for the three-dimensional structure of aspartic proteinases from family A1 hold true for pepsin A. Its N-terminal domain comprises residues 1 – 170, whereas the C-

terminal domain encompasses residues 171 – 326. In simple terms, a β-sheet consisting of two layers extends over each domain. A further six-stranded sheet spans the two domains. Pepsin A is composed of just a few and short helices (see Figure 1.3). Some of them are often referred to as helical-type regions, because they contain less than two turns (Andreeva, *et al.*, 1984; Cooper, *et al.*, 1990).

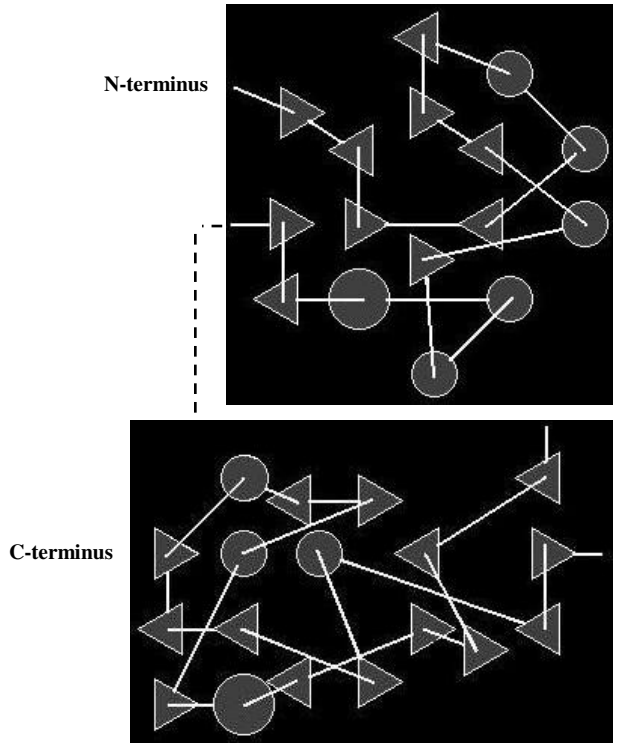

Figure 1.3 – Topology of porcine pepsin A. Cartoon generated upon submission of Cartesian coordinates of atoms in the molecule of monoclinic porcine pepsin A (PDB code *4pep*) to the algorithm TOPS (Michalopoulos, *et al.*, 2004), which can be accessed at http://www.tops.leeds.ac.uk and is maintained by the *Universities of Glasgow* and *Leeds*. The software yielded individual diagrams for each domain, which were subsequently connected through a discontinuous line. In the illustration, triangular symbols represent β-strands, whilst circular symbols stand for helices. Upward and downward pointing triangles respectively depict 'up' and 'down' strands. Each secondary structural element is directed from the N- to C-termini. If the N-terminal connecting line is drawn to the edge and the C-terminal one to the centre of the symbol, the direction is up. On the other hand, whenever the N-terminal connection is drawn to the centre and the C-terminal one to the edge of the symbol, the direction is down.

In spite of being topologically similar, the N- and C- terminal domains differ in several aspects, such as amino acid composition, disulphide-bonding pattern and local flexibility. Three out of four basic residues and four out of six cysteine residues belong to the C-terminal domain. The chain of pepsin A is cross-linked by three disulphide bridges. The first one is localized after the catalytic Asp32 and the second one precedes the catalytic Asp215. These disulphide linkages form two loops which comprise four and three residues, respectively. On the other hand, the loop formed by the third disulphide bridge embraces 32 residues of the second half of the C-terminal domain (Tang, *et al.*, 1973). Lastly, the average *B*-factor of residues belonging to the C-terminal domain is about two-fold higher than that of the residues in the N-terminal domain (Sielecki, *et al.*, 1990; see Figure 1.4).

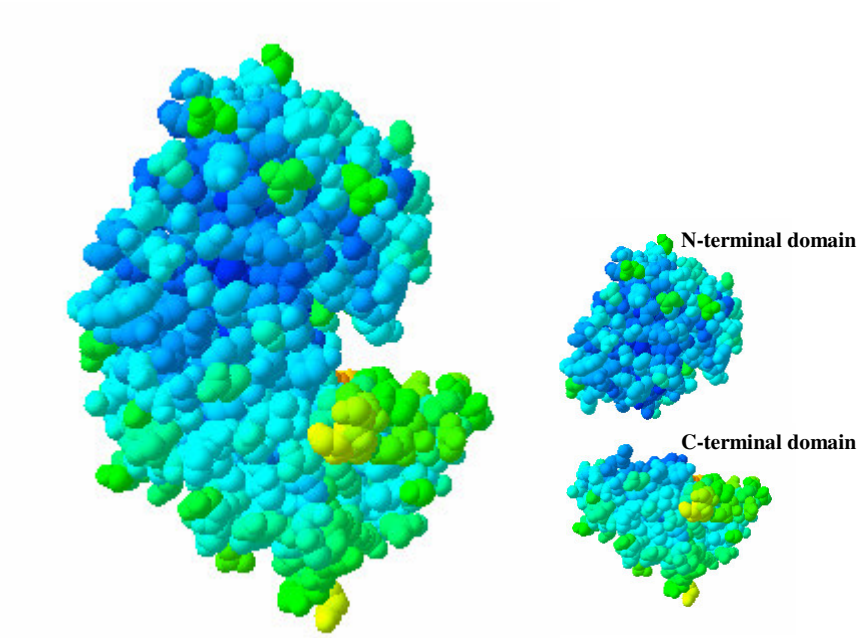

Figure 1.4 – Flexibility of porcine pepsin A (PDB code *4pep*). Residues are coloured according to *B*-factor. The colour scheme includes spectral colours: on one end, dark blue stands for low *B*-factor; on the opposite end, red stands for high *B*-factor. These figures were generated and rendered by means of the interface *Swiss-PdbViewer 3.7*.

B.3. *Catalytic Properties: Specific Considerations*

Pepsin A is highly stable at low pH as compared to other proteins, including other aspartic proteinases. Optimal pH for its enzymatic activity is dependent on whether the substrate is a protein or a peptide. Hydrolysis of proteins catalyzed by this aspartic proteinase is generally maximal within the pH range from 1 to 2 (Baker, 1951; Cornish-Bowden & Knowles, 1969; Pletschke, *et al.*, 1995). On the other hand, cleavage of synthetic peptides was found to be on average optimally catalyzed by pepsin A at higher pH values (within the 2.0 – 4.2 pH interval) (Baker, 1951; Kotler, *et al.*, 1989; Lin *et al.*, 1992b; Martin, 1984; Pletschke, *et al.*, 1995). Spontaneous acquirement of a disordered conformational state and instantaneous abolishment of enzymatic activity follow from exposure of this aspartic proteinase to pH values above 7 (Campos & Sancho, 2003; Lin, *et al.*, 1992b; Lin, *et al.*, 1993; Pletschke, *et al.*, 1995).

Each catalytic aspartic residue of pepsin A is contained in the sequence motif Asp – Thr – Gly (Tang, *et al.*, 1973). The aspartic dyad has been surmised to consist of a biological requisite for intramolecular activation and optimal activity *in vivo* under extreme pH conditions (Sinkovits, *et al.*, 2006).

In agreement with its biological function, pepsin A has relatively broad specificity (Dunn, *et al.*, 1986). However, it exhibits main preference for cleavage between amino acid residues containing at least one hydrophobic or aromatic side chain, such as phenylalanine, tyrosine, leucine and methionine (Dunn & Hung, 2000; Fruton, 1970).

CHAPTER II

OBJECTIVES & OUTLINE

This chapter is devoted to the general objectives of the current dissertation and the approach adopted to accomplish them.

In order for a protein to adequately perform its function under specific *in vivo* or *in vitro* environmental conditions for a sufficient period of time, preservation of its chemical and conformational integrity becomes crucial. Actually, deficient stability imposes restrictions in industrial, biotechnological and medical applications of proteins. Therefore, attempts to enhance protein stability have incited interest in surveying the mechanisms underlying such biophysical property.

Owing to its major physiological role in hydrolyzing acid-denatured proteins in stomach, pepsin A is an acidophilic protein. High enzymatic activity at extremely low pH values entails fine-tuning of protein stabilization strategies, which confer high resistance to such harsh conditions. Nonetheless, pepsin A undergoes an order-to-disorder transition in a narrow near-neutral pH range with deleterious consequences on its activity (Campos & Sancho, 2003; Kamatari, *et al.*, 2003), so as to prevent tissue damages on stomach and duodenum due to a continuous proteolytic action (Allen & Flemström, 2004; Carginale, *et al.*, 2004). A large number of studies focused on the pH-assisted denaturation reaction of pepsin A (Bryksa, *et al.*, 2003; Campos & Sancho, 2003; Dee, *et al.*, 2006; Favilla, *et al.*, 1997; Konno, *et al.*, 2000; Lin, *et al.*, 1993; Makarov, *et al.*, 1991; Tanaka & Yada, 2001) have provided insights into its acidostability and instability at neutral and alkaline pH.

Mechanisms accountable for innate robustness of pepsin A in highly acidic media do not necessarily afford its resistance to other environmental setups. Indeed, thorough understanding of the conformational stability of a protein is solely attainable through exploration of the influence of diverse denaturing factors on its natively folded state. This may be rationalized by the fact that different strategies for perturbing proteins emphasize the roles of different types of interactions and molecular features in the stabilization of the folded conformational state. Aside from modifications on pH, other methods have been employed in order to promote and investigate pepsin denaturation, namely changes in temperature (Privalov, *et al.*, 1981; Tello-

Solís & Romero-García, 2001) and pressure (Fujiwara *et al.*, 2001), or addition of chaotropes (Ahmad & McPhie, 1978; Ahmad & McPhie, 1979; Blumenfeld, *et al.*, 1960; McPhie, 1980; McPhie, 1989; Perlmann, 1959) and organic solvents (Dalgalarrondo, *et al.*, 1995; Dharmapuri & Saiprakash, 1999; Kang, *et al.*, 1994a; Kesavulu, *et al.*, 2005; Tang, 1965). In a large number of studies, however, the effects of the mentioned denaturing factors on pepsin A have been assessed: i) at pH values outside the range within which the protein assumes its native state and exhibits optimal catalytic activity; or ii) upon inactivation by reaction with inhibitors. Hence, inferences on the conformational stability of the natively folded state of pepsin A from data obtained on these studies are not straightforward.

Premised on the idea of filling gaps on the conformational stability of native pepsin A, this research primarily aims at elucidating the effects of increasing concentrations of denaturants, precisely, acetonitrile, guanidine hydrochloride and urea, on its folded and fully active state under physiological-like conditions. A few lateral experiments will allow adding some knowledge on pH- and temperature-induced denaturation of the target protein.

The concern with providing an experimental setup comparable to the *in vivo* environment where the protein under study performs its biological function, so that the biomacromolecule adopts its unique native state, is rationalized by the widespread assumption that such conformational state is, in general, the most stable among the ensemble of all possible states or, at least, among the ensemble of kinetically accessible states (Creighton, *et al.*, 1996; Govindarajan & Goldstein, 1998). Consequently, one could regard the conformational search for the natively ordered state as a pursuit for an optimized balance of forces and structural features which fulfils the requirement of energy minimization. And, such set of specific physical and molecular factors, which determines favourableness of the native state in detriment of other conformational states *in vivo*, should be a major focus of interest of protein stability engineers.

Dependence of conformational integrity of natively folded porcine pepsin A on environmental conditions will be monitored by assessment of intrinsic spectral and hydrodynamic properties, thermal stability and enzymatic activity, after fixed periods of exposure to physical and chemical denaturing factors.

Appraisal of changes promoted by the elected denaturants will allow delineating denaturation pathways of the natively ordered state of porcine pepsin A under selected experimental conditions, and identifying gross characteristics of non-native states sampled by the protein along the course and in the end of induced conformational transitions. Comparative analyses of results obtained for the impact of the three denaturants on the protein through the outlined experimental approach, in conjunction with updated information on the mechanism of action of each chemical agent on proteins in general, will hopefully shed some light into the foundations of the conformational stability of the protein under investigation. Conclusions withdrawn from this research will be reconciled with information, available at open literature, on denaturation and conformational stability of pepsin A in different experimental setups.

Despite extensive evidence on the ability of acetonitrile, guanidine hydrochloride and urea to induce structural perturbation on proteins; molecular and physical bases of the action of these denaturants and the contributions from different forces and molecular features of proteins to their sensitivity / resistance against

these agents are still under discussion. Pepsin A seems to be an appropriate means for giving, at least, some speculative insights into these issues. In fact, a wealth of experimental data on the activation processes and structure of its zymogen, structures of the mature and inhibited forms, enzyme specificity and kinetics, catalytic and inhibition mechanisms (Andreeva, *et al.*, 1984; Chen, *et al.*, 1992; Fruton, 1970; Fruton, 2002; Hartsuck, *et al.*, 1992; Marciniszyn, *et al.*, 1976; Pohl & Dunn, 1988; Sielecki, *et al.*, 1990) has withstood the utilization of pepsin A as a model system for diverse studies, not only on structure, function and their interrelationships, but also on protein stability (Lin, *et al.*, 1989; Yoshimasu, *et al.*, 2004). In the last analysis, unusual properties of pepsin A readily encourage one to use it as an instrument for addressing fundamental problems in protein science.

CHAPTER III

MATERIALS & METHODS

In the following sections, material, equipment and procedures used throughout the research conducive to this dissertation will be described.

1. *MATERIALS*

A. *Proteins, Substrates and Amino Acids*

Pepsin A from porcine gastric mucosa, N-acetyl-L-tryptophanamide (NATA) and glycine were purchased from *Sigma*. The synthetic peptide H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe.TFA (M_r = 835.88) was a product of *Bachem.*

B. *Salts, Solvents and Other Chemicals*

Dimethyl sulphoxide (DMSO), trifluoroacetic acid (TFA), sodium acetate, sodium chloride, ammonium bicarbonate, citric acid, disodium hydrogen orthophosphate, guanidine hydrochloride and urea were of analytical reagent grade, and were used as received. Acetonitrile was of HPLC grade.

Details on other chemicals and kits will be supplied elsewhere.

2. *METHODS*

A. *Preparation of Solutions*

Buffers and other solutions were prepared using ultra-pure water. pH was measured with a *Hanna* microprocessor, which used to be calibrated daily. Concentrated solutions of chloridric acid and sodium hydroxide were utilized to adjust pH.

Fresh concentrated stocks of guanidine hydrochloride and urea were made from weighed quantities of these chemical compounds in 10 mM sodium acetate at pH 3. Slow decomposition of urea solutions with subsequent formation of cyanate and ammonium ions is well-known. Accumulation of cyanate ions can cause unwanted carbamylation of proteins, giving rise to modifications in protein structure, stability and function (Lin, *et al.*, 2004). With the aim of circumventing such side effects, glycine was added to denaturant stocks at a final concentration equal to 5 mM, in case incubation of porcine pepsin A in aqueous solutions of urea lasted longer than 1 h. In a solution of urea and glycine, cyanate ion acts as a carbamoylating agent in the conversion of glycine into *N*-carbamoyl glycine (Shapiro, 1999).

Phosphate-citrate buffers at different pH values (from pH 3 to 8) were prepared from solutions of disodium hydrogen orthophosphate at 0.2 M and citric acid at 0.1 M, according to standard procedures.

B. *Preparation of Stocks of Porcine Pepsin A*

Porcine pepsin A commercially available is purified by crystallization followed by chromatography, and it is supplied as a lyophilized powder. The protein was stored at -20ºC, and used without further purification.

Depending on the requirements of each technique, stocks of porcine pepsin A at different concentrations were prepared. The protein was dissolved in 10mM sodium acetate, titrated to pH 3 with a solution of chloridric acid. Several aliquots of each concentrated stock were stored at -20ºC for short periods of time, so as to avoid eventual, unwanted denaturation and autolysis, and subsequent loss of enzymatic activity. According to Qiao and co-workers (2002), the higher the concentration of porcine pepsin A during storage, the longer its half-life.

C. *Quantification of Solutions of Porcine Pepsin A*

Protein concentration was assessed by means of spectrophotometric assays.

C.1. *Ultraviolet Absorption Spectroscopy*

The procedure regularly used consisted of measuring an absorption spectrum in the ultraviolet (UV) region (in the range from 250 to 340 nm) for each protein sample, or for each dilution of a protein stock (in duplicate), in a double-beam spectrophotometer *Jasco V-530 UV/VIS*. Measurements were performed using adequately matched quartz cells with 1-cm path length. The reference cell was filled with 10 mM sodium acetate at pH 3. Each 'difference' spectrum was recorded at 40 nm/min, and it was the average of three scans. Parameters such as bandwidth and data pitch were set to 2 and 0.5 nm, respectively.

Light scattering may turn out to be a significant interference in spectrophotometric measurements of protein concentration due to, for instance, the presence of large particles in solution, such as aggregates or turbidity at higher protein concentrations. This leads to an artefactual increase in the absorbance within the absorption envelope. Unless a protein contains a prosthetic group, a clear protein solution will normally show no absorbance at wavelengths greater than 310 nm (Lewis, 1992; Mantle & Harris, 2000; Schmid, 2005). With the purpose of correcting for eventual contributions from light scattering, the value of absorbance at 310 nm was subtracted from the absorbance reading at 280 nm.

Using an extinction coefficient of 38,600 $M^{-1}.cm^{-1}$ at 280 nm (Aoki, *et al.*, 1997), protein concentration was calculated from the Beer-Lambert relationship, mathematically expressed as (John, 1992):

$$A(Y) = \varepsilon_Y * C * l \qquad (3.1),$$

where A(Y) denotes absorbance at Y nm, ε_Y stands for the extinction coefficient at Y nm ($M^{-1}.cm^{-1}$), C corresponds to the protein concentration (M) and l represents the cell path length (cm).

C.2. *Micro BCA™ Protein Assay Reagent Kit*

Sporadically, *Micro BCA™ Protein Assay Reagent Kit* (*Pierce*), which is based on a colorimetric method, was utilized for confirming concentrations of extremely dilute protein samples. The detection sensitivity of this commercial kit is of 0.5 – 20 μg/mL.

A set of dilute bovine serum albumin (BSA) standards at different protein concentrations and *Micro BCA* working reagent were prepared according to kit instructions. 500 μL of *Micro BCA* working reagent were added to each dilute sample of porcine pepsin A (with 500 μL of final volume). After consecutive incubations of 1 h at 60ºC and 40 min at 25ºC, absorbance at 562 nm was measured for every standard and sample replicate.

Absorbance readings were blank-corrected by subtracting the average absorbance value corresponding to blank standard replicates. Per each assay, a curve was generated by plotting absorbance readings at 562 nm of each standard *versus* its known concentration. After ensuring the linearity of the entire

standard curve, unknown concentration of porcine pepsin A in each sample was determined from this plot.

D. *Assessment of Purity and Autolysis of Porcine Pepsin A*

Regularly, purity and structural integrity of the enzyme were determined by vertical electrophoresis in polyacrylamide gels under denaturing (SDS - PAGE) and near-native conditions. Gels were polymerized in a *Hoefer Mighty Small SE 245* dual gel caster (*Amersham Biosciences*), and electrophoreses were performed in a *Hoefer SE 250 Mighty Small II* unit (*Amersham Biosciences*).

D.1. *Polyacrylamide Gel Electrophoresis*

D.1.1. *Polyacrylamide Gel Electrophoresis under Denaturing Conditions (SDS - PAGE)*

The procedure adopted for sodium dodecyl sulphate - polyacrylamide gel electrophoresis is based on the Laemmli system (Laemmli, 1970), which is the most common protocol for sodium dodecyl sulphate - denatured proteins. Samples were run in a discontinuous gel / buffer system, composed of a stacking gel and a separating gel at different concentrations of acrylamide:bisacrylamide, and containing Tris buffers at different salt concentrations and pH. In this system, proteins were concentrated in the stacking gel, and electrophoretically separated in the separating gel (Goldenberg, 1990).

The instructions ahead were followed for preparation and run of a single discontinuous gel / buffer system.

Preparation of the Separating Gel

Concentration of acrylamide:bisacrylamide in the separating gel – 15% (w/v) – was selected taking into account the molecular mass of the protein of interest.

The solutions listed below were successively added in the same order as indicated:

(i) 1,300 μL of ultra-pure water;
(ii) 1,675 μL of 1.5 M Tris at pH 8.8;
(iii) 1,875 μL of 40% (w/v) acrylamide:bisacrylamide;
(iv) 100 μL of 10% (w/v) sodium dodecyl sulphate.

The mixture was smoothly homogenized. Immediately before pouring the gel solution, 50 μL of 10% (w/v) ammonium persulphate (polymerization initiator) and 2.5 μL of N, N, N', N'-tetramethylene-ethylenediamine (for short, TEMED – a polymerization catalyst) were added, and mixed by gently inverting the container.

Rapidly, the assembled gel plates were filled with 3,500 µL of the separating gel solution, taking care no to trap any air bubbles within the gel. The surface of the separating gel solution was carefully layered with 50% (v/v) isopropanol, so as to prevent gel exposure to oxygen and to keep the gel surface flat.

Finally, the separating gel was allowed to rest for 1 h in order to polymerize. After the gel had polymerized, isoproponal was decanted off, and drained on tissue paper.

Preparation of the Stacking Gel

Similarly to the separating gel, the solutions listed ahead were successively added, in the same order as indicated, for preparing the stacking gel at 4% (w/v) acrylamide:bisacrylamide:

(i) 1,495 µL of ultra-pure water;
(ii) 625 µL of 0.625 M Tris at pH 6.8;
(iii) 244 µL of 40% (w/v) acrylamide:bisacrylamide;
(iv) 50 µL of 10% (w/v) sodium dodecyl sulphate.

The mixture was smoothly homogenized. Immediately before pouring the gel solution, 25 µL of 10% (w/v) ammonium persulphate and 1.25 µL of TEMED were added, and mixed by gently inverting the container.

The stacking gel solution was transferred onto the separating gel until it reached the top of the smaller plate. A comb was immediately inserted, preventing entrapment of air bubbles on the ends of the teeth.

At last, the stacking gel was left for 40 min in order to completely polymerize.

Preparation of Samples

Denaturing solution, composed of 2% (v/v) 2-mercaptoethanol, 0.1 M Tris, 0.1 M bicine, 2% (w/v) sodium dodecyl sulphate, 8 M urea and 0.0015% (w/v) bromophenol blue, was added in a 1:1 (v/v) proportion to each protein sample. Bromophenol blue is a tracking dye which helps to monitor sample application.

Samples were heated at 100ºC in a water bath for 5 min. Afterwards, they were cooled at room temperature, and spinned down.

Loading of Samples

The gel sandwich was placed in the electrophoresis unit. The running buffer [0.1 M Tris, 0.1 M bicine, 0.1% (w/v) sodium dodecyl sulphate] was added, so that both the top and the bottom of the gel were immersed in running buffer. The comb was carefully removed.

Samples were loaded into the gel wells with a 25 µL *Hamilton* microsyringe, which was thoroughly rinsed with running buffer after introduction of each sample.

Electrophoretic Run

Electrophoresis was carried out at constant voltage (125 V) for 95 min.

During the run, heating of the electrophoresis apparatus was avoided.

D.1.2. *Polyacrylamide Gel Electrophoresis under Near-Native Conditions*

A procedure for polyacrylamide gel electrophoresis in the absence of denaturants was straightforwardly adopted from the one just described for sodium dodecyl sulphate - polyacrylamide gel electrophoresis. It follows that samples were also run in an analogous discontinuous gel / buffer system. Absence of denaturants in the gel / buffer system and samples is the main difference between the two methodologies. pH values of the running buffer and of the buffers composing the gels were maintained. Recalling that porcine pepsin A undergoes denaturation at neutral-alkaline pH values, polyacrylamide gel electrophoresis did not occur exactly under native conditions; even though sample preparation involved neither addition of a denaturing solution nor heating.

Preparation of the Separating Gel

The separating gel at 15% (w/v) acrylamide:bisacrylamide was prepared by sequential addition of the chemicals listed below, in the same order as indicated:

(i) 1,400 μL of ultra-pure water;
(ii) 1,675 μL of 1.5 M Tris at pH 8.8;
(iii) 1,875 μL of 40% (w/v) acrylamide:bisacrylamide.

The mixture was smoothly homogenized. 50 μL of 10% (w/v) ammonium persulphate and 2.5 μL of TEMED were added, and mixed by gently inverting the container, immediately before pouring the gel solution.

Assembled gel plates were filled right away with 3,500 μL of the separating gel solution. The surface of the separating gel solution was covered by 50% (v/v) isopropanol.

At last, the separating gel was allowed to rest for 1 h in order to polymerize. Isoproponal was poured out, and drained on tissue paper, after complete gel polymerization.

Preparation of the Stacking Gel

The solutions listed ahead were successively added, in the same order as indicated, for preparing the stacking gel at 4% (w/v) acrylamide:bisacrylamide:

(i) 1,545 μL of ultra-pure water;
(ii) 625 μL of 0.625 M Tris at pH 6.8;

(iii) 244 μL of 40% (w/v) acrylamide:bisacrylamide.

The mixture was smoothly homogenized. Immediately before pouring the gel solution, 25 μL of 10% (w/v) ammonium persulphate and 1.25 μL of TEMED were added, and mixed by gently inverting the container.

The stacking gel solution was transferred onto the separating gel, and a comb was immediately inserted. It was left for 40 min, in order to completely polymerize.

Preparation of Samples

Loading solution, composed of 50% (v/v) glycerol and 0.008% (w/v) bromophenol blue, was added to each protein sample. Glycerol facilitated loading of the protein sample under the running buffer level. The ratio of loading solution to protein sample was 1:4 (v/v).

Loading of Samples

After being placed in the electrophoresis unit, the gel sandwich was completely immersed in running buffer [0.1 M Tris, 0.1 M bicine], and the comb was carefully removed.

Samples were applied in the gel wells with a 25 μL *Hamilton* microsyringe, which was thoroughly rinsed with running buffer before introduction of different samples.

Electrophoretic Run

After 15 min at 6 mA, current was increased, so that the gel was constantly submitted to 20 mA for 210 min.

Electrophoresis apparatus was cooled during the run, so as to circumvent problems associated with excessive heating.

D.2. *Protein Staining on Electrophoresis Gel*

Silver nitrate staining is a popular method for detecting protein bands within an electrophoresis gel, owing to its high sensitivity. It can detect as little as 2 ng of a protein in a single band (Bollag & Edelstein, 1994).

Gels were silver-stained according to a procedure developed by O'Connell and Stults (1997). Precise timing, constant temperature and gentle agitation (35 rpm) were assured. Fingerprints and surface drying were avoided, so as to minimize surface artefacts (Bollag & Edelstein, 1994).

Proteins in the gel were fixed for 30 min in 30% (v/v) ethanol and 10% (v/v) acetic acid. This treatment was repeated for three times. In the fixing step, acid prevented diffusion of the biomacromolecules out of the gel, by rendering them insoluble.

Afterwards, the gel was shacked in 20% (v/v) ethanol for 10 min, and rinsed for another 10 min in ultra-pure water. This step allowed substances in the gel interfering with silver nitrate to be washed out.

Subsequently, the gel was treated with 0.2 g/L sodium thiosulphate. This is the so-called sensitization step, whereby proteins were chemically modified and rendered more reactive towards silver. After 1 min, the gel was washed twice for 20 sec in ultra-pure water, for excess of sodium thiosulphate leads to a high level of background staining.

The following step consisted of silver impregnation. The gel was incubated for 30 min in 2 g/L silver nitrate. This compound was the source of silver ions. Rinsing the gel in ultra-pure water for 5 to 10 sec permitted removal of excess of silver ions from the surface.

A solution composed of 0.7 mL/L formaldehyde (37%), 30 g/L sodium carbonate and 10 mg/L sodium thiosulphate was employed for the development of the gel. Formaldehyde served as a reductant to convert silver ion into metallic silver. Sodium carbonate shifted the pH to approximately 12, facilitating the reduction process. Incubation of the gel in the developing solution lasted until the bands became visible.

To stop the development, the gel was immersed in a solution containing 50 g/L Tris and 2.5% (v/v) acetic acid for 1 min. The stopping solution prevented further reduction of silver ions.

D.3. *Analysis, Preservation and Storage of Electrophoresis Gels*

Gels were scanned on a *FX-710* (*Bio-Rad*) densitometer, and analysed with the *Quantity One* software (*Bio-Rad*). They were conserved in ultra-pure water, and stored on plastic packages at 4ºC.

E. *Induction of Porcine Pepsin A Denaturation*

As it was abovementioned, the object of this study was native porcine pepsin A. Consequently, choice of pH for the protein solvent (10 mM sodium acetate) was premised on the need to provide the proton concentration at which the protein exhibits conformational stability close to that displayed under physiological conditions. Luminal pH is around 2. Nonetheless, selection of pH 3 was assumed to fulfil this condition, in line with data from Campos and Sancho (2003). These authors concluded that pepsin maintains a natively folded conformational state until about pH 3.5. Besides, the higher the pH the lower the propensity for autolysis (Glazer & Smith, 1961; Qiao, *et al.*, 2002).

Chemical-induced denaturation of porcine pepsin A involved its incubation in the presence of growing concentrations of denaturants (acetonitrile, guanidine hydrochloride and urea). For this purpose, a serial method was adopted. Stocks of denaturants were diluted with 10 mM sodium acetate at pH 3 to give a series of solutions of constant volume and at a wide range of concentrations. An aliquot of fixed volume and concentration of porcine pepsin A was added to each solution of denaturant. Assays were performed in which a set of pepsin samples was incubated in a mixture of two denaturants: while one was maintained at constant concentration, the concentration of the other varied from one sample to another.

In respect of pH-promoted denaturation of porcine pepsin A, it entailed direct dissolution of the protein and its incubation in phosphate-citrate buffers at different pH values (from pH 3 to 8).

Incubation took place in non-stirred conditions at the constant temperature value of 25ºC. It is believed that vigorous stirring of solutions of pepsin results in surface denaturation (Williams & Rajagopalan, 1966). Although the enzyme under study was isolated from a mammal and *in vivo* temperature conditions should be around 37ºC; 25ºC is the temperature condition most often used in biochemistry. As it shall be demonstrated later, there should be no meaningful differences in structural terms on submission of porcine pepsin A to 25 or 37ºC. Moreover, the odds for autolysis should be lessened at lower temperature values. Finally, 25ºC is a temperature value easily maintained resorting to thermostatically controlled heating, since it is very close to room temperature. For the majority of the experiments, incubation phases lasted precisely 1 h. In a few cases, the incubation period was shortened or extended (up to a maximum of 3 days).

F. *Measurement of Spectroscopic Properties of Porcine Pepsin A*

F.1. *Circular Dichroism Spectroscopy*

In the current study, steady-state circular dichroism spectroscopy in the amide region was employed to report on changes occurring in the backbone structure of porcine pepsin A. Modifications in the tertiary structure of the aspartic proteinase were detected by following circular dichroic signals in the aromatic region.

Most near- and far-UV circular dichroic spectra of porcine pepsin A were generated in a *Jasco J-810* spectropolarimeter. As it shall be indicated ahead, a few spectra were recorded in a *Jasco J-600* spectropolarimeter. *Spectra Manager* software for *Windows* from *Jasco* was used to select circular dichroism parameters, to control the recording process and to handle the spectra.

F.1.1. *Measurements under Isothermal Conditions*

Concentration of porcine pepsin A was typically in the range from 12.686 to 14.286 μM during the phases of incubation and data collection.

Measurements of circular dichroic signal in the far-UV region were accomplished in a cylindrical, quartzo cell with 0.02-cm path length, and in the wavelength range from 185 to 260 nm. Circular dichroic spectra in the near-UV region were acquired over the wavelength interval from 260 to 340 nm, using a rectangular, quartzo cell with 1-cm path length. Bandwith and data pitch were set to 1 nm and 0.2 nm, respectively. For each spectrum, six to eight consecutive scans were obtained at 50 nm/min of scanning

speed. Repetitive scanning was used to obtain an optimal signal-to-noise ratio (Schmid, 1990).

High tension (HT) voltage was continuously recorded during collection of circular dichroic spectra. Monitoring of high tension voltage is useful for detecting sample abnormalities. For example, an excessive high tension voltage may be indicative of high absorbance by the sample (Rodger & Ismail, 2000).

Each raw spectrum was corrected for the corresponding background, smoothed and normalized (outside the absorption envelope). Measured ellipticity (θ) was converted into mean residue ellipticity ($[\theta]_{MRW}$, in deg/cm^2/dmol) by using the equation (Schmid, 1990):

$$[\theta]_{MRW} = (\theta * 100 * MRW) / (c * l) \quad (3.2),$$

where θ is the observed ellipticity (deg), c is the protein concentration (mg/mL), l is the path length (cm), and MRW stands for the mean residue weight (or mass). A mean residue weight of 107 Da was used in the calculation of mean residue ellipticity (Yoshimasu, *et al.*, 2004).

F.1.2. *Measurements in Thermal Gradients*

Porcine pepsin A was incubated at 14.286 μM, in the presence of selected concentrations of denaturant, and under the experimental conditions described in the item '*Induction of Porcine Pepsin A Denaturation*'. Immediately before data collection, each protein sample was submitted to a 1:2.5 dilution with corresponding incubation medium, due to technical requirements.

A circular dichroic spectrum of a protein sample was recorded after equilibration for 5 min at every 2ºC increment in the temperature value. Spectra were collected in the range from 25 to 99ºC. The spectropolarimeter was equipped with a temperature-controlled sample holder, an external circulating heater and a stirrer, in order to provide and precisely maintain the required temperature conditions for the sample. Circular dichroic signal was acquired in a 0.1-cm path length cell, and in the wavelength range from 195 to 260 nm. Bandwith and data pitch were set to 1 nm and 0.2 nm, respectively. Each reported spectrum corresponded to the accumulation of four consecutive scans performed at 50 nm/min.

Spectra were processed according to the procedure described above, under the heading '*Measurements under Isothermal Conditions'*. In addition, thermal transition temperature values were estimated from plots of mean residue ellipticity against temperature.

F.1.3. *Estimation of Secondary Structure from Far-UV Circular Dichroic Spectra*

Relative amounts of secondary structural elements of porcine pepsin A were computed from corresponding circular dichroic spectra in the amide region, resorting to two different computer programs, *Selcon3* (*the self-consistent method for protein CD analysis*, version 3) (Sreerama & Woody, 1993) and

ContinLL (Provencher & Glockner, 1981) available at the online server *DICHROWEB* (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) (Lobley, *et al.*, 2002; Whitmore & Wallace, 2004). The algorithms rely on reference datasets of proteins with known secondary structure and circular dichroic spectra, rather than on typical spectra for each type of secondary structure motif. High tension voltage accompanying acquisition of submitted spectra was ensured not to exceed 550 mV at 190 nm; otherwise, reliability of output data could be compromised.

F.2. *Intrinsic Tryptophanyl Fluorescence Spectroscopy*

Intrinsic, tryptophanyl, steady-state fluorescence spectroscopy was accomplished in order to monitor perturbations in the tertiary structure of porcine pepsin A, a multitryptophan protein containing tyrosine and phenylalanine residues.

Appraisal of the contribution of tryptophan residues to intrinsic fluorescence of porcine pepsin A was performed in a *Perkin Elmer LS 50B* luminescence spectrometer, interfaced to a computer. Instrumental set up and operation of the spectrofluorophotometer were accomplished by the software *Fl WinLab* from the supplier. A rectangular, quartz cell with 1-cm path length and polished surfaces on all four sides was used.

Concentration of porcine pepsin A was kept at 2.857 μM during the incubation phase and the recording of fluorescence spectra. Absorbance reading of protein samples at the excitation wavelength was assured to be lower than 0.1, in order to minimize inner filter errors (Szabo, 2000). Fluorescence measurements of the free tryptophan residue, NATA, were preceded by an incubation stage during which it was in the same experimental setup as described for the protein. Concentration of NATA was kept at 22.1 μM throughout the period of incubation and during the recording of fluorescence spectra.

Excitation was performed at 295 nm with the purpose of selectively detecting tryptophanyl emission and alleviating interference from tyrosine residues. Tyrosine residues barely show absorbance at 295 nm (Schmid, 1990; Schmid, 2005; Szabo, 2000). Fluorescence emission spectra (from 300 to 400 nm) of a maximum of four replicates of each sample of porcine pepsin A and NATA were recorded at a scan speed of 100 nm/min. Slit widths of both excitation and emission monochromators were set to 2.5 nm. Each spectrum corresponded to an average of three scans. During the measurements, the cell was covered by a lid.

Reference spectra recorded under identical instrumental conditions were subtracted from raw spectral data. Analysis of subtracted spectra focused on the position of the spectrum, *i. e.*, the wavelength corresponding to the maximum intensity of fluorescence emission (λ_{max}).

G. *Size Exclusion Chromatographic Analyses of Porcine Pepsin A*

Analytical size exclusion liquid chromatography was employed as a strategy for following changes

in hydrodynamic properties of porcine pepsin A, upon exposure to denaturing factors, from its elution behaviour.

Samples of porcine pepsin A were prepared and incubated as described under the heading '*Induction of Porcine Pepsin A Denaturation*'. Typical protein concentration was equal to 42.857 μM. It is worth adding that samples containing visible aggregates were centrifuged at 14,000 rpm and 25ºC for 1 min, immediately before application to the chromatographic column. In those cases, only the supernatant was collected and analyzed, in order to prevent matrix blockage.

100 μL - samples were loaded onto a previously equilibrated analytical size exclusion chromatographic column, *Superdex 75 HR 10/30*, by means of a 250 μL *Hamilton* microsyringe. The chromatographic column, whose matrix was composed of dextran covalently bound to highly cross-linked agarose, was coupled to a FPLC system, an *ÄKTA*^{TM} *basic*, both purchased from *Amersham Biosciences*. The chromatographic system was controlled by *Unicorn* software from the supplier. Porcine pepsin A was eluted at a flow rate of 1 mL/min with an isocratic gradient of the same medium as that used for the incubation step. A minimum of two replicates per each solvent condition tested under study were analysed. Eluents were filtered through a membrane with 0.2-μm pore, in order to save the column from being blocked by undissolved compounds, and degassed with helium prior to use.

A low-range molecular mass kit from *Amersham Biosciences* was utilized for calibration of the size exclusion chromatographic column. The calibration kit consisted of the following proteins: albumin from bovine serum (67,000 Da), ovalbumin from hen egg (43,000 Da), chymotrypsinogen A from bovine pancreas (25,000 Da), and ribonuclease A from bovine pancreas (13,700 Da). Calibration standards were, to a good approximation, globular in shape (as cited in *Gel Filtration Calibration Kits* Instructions, *Amersham Biosciences*, 2002) just like the protein under study. The kit also included Blue Dextran 2,000, which was applied to the column in order to determine the void volume (V_0). A molar mass of approximately $2*10^{+6}$ and a Stokes radius of 350 Å are ascribed to Blue Dextran 2,000. Protein calibrants and Blue Dextran 2,000 were prepared and run according to instructions provided by the supplier (*Gel Filtration Calibration Kits* Instructions, *Amersham Biosciences*, 2002).

Effluent was continuously monitored by recording absorbance at 220 and 280 nm. Chromatograms were processed using the software abovementioned, so as to determine elution volumes (V_e) and peak areas.

A molecular mass calibration curve was built by plotting the *gel-phase distribution coefficient*, K_{av}, *versus* the base-10 logarithm of the molecular mass of each calibration standard (in accordance with the *Gel Filtration Calibration Kits* Instructions, *Amersham Biosciences*, 2002). The elution parameter K_{av} was computed from the following equation (Scopes, 1994; Zeev-Ben-Mordehai, *et al.*, 2003):

$$K_{av} = (V_e - V_0) / (V_t - V_0) \qquad (3.3),$$

where V_e corresponds to the elution volume for the protein, V_0 is the column void volume (taken as the elution volume for Blue Dextran 2,000), and V_t stands for the total bed volume. K_{av} for a protein defines the proportion of pores that can be occupied by the protein of interest (Scopes, 1994).

H. *Differential Scanning Calorimetric Assays of Porcine Pepsin A*

In a differential scanning calorimetric assay, heat effects associated with structural transformations of a protein induced by changing temperature are assessed. Notwithstanding temperature is the principal independent variable, other denaturing agents are often used as second independent variables. Porcine pepsin A pre-equilibrated in different solvent conditions was submitted to differential scanning calorimetric analyses, in order to judge the effect of denaturants and pH on thermal stability.

Differential scanning calorimetric assays were conducted on a high-sensitivity *MicroCal VP-DSC* calorimeter, interfaced to a computer. Equipment and scans were controlled by *Origin DSCITC* software (*MicroCal*).

In agreement with the guidelines previously provided in the section 2.E. '*Induction of Porcine Pepsin A Denaturation*', samples at a protein concentration typically around 5.714 μM were prepared and incubated at 25ºC. Samples and references were degassed before loading, so as to prevent microscopic bubble formation as temperature was raised. Solutions were carefully loaded into the corresponding hermitically sealed cells of the calorimeter by means of a syringe. Exhaustive cleaning of the cells was undertaken before each assay. After a thermal equilibration phase of 5 min, a sample was heated alongside its matching reference at a constant scan rate of 60ºC/h until a maximum temperature value of 100ºC was attained. Calorimetric data were collected under constant external pressure, in order to avoid both bubble formation and evaporation at high temperature values. As the temperature increased, differences in the heat energy uptake by sample and reference were recorded. A maximum of three replicates per solvent condition were tested. Reversibility of thermal denaturation reactions was checked sporadically by examining the reproducibility of the calorimetric trace in a second heating of the sample, after the completion of the thermal transition and cooling from the first scan.

Heat capacity thermograms were handled using the instrumental software. Correction for differences between cells was accomplished by subtracting reference scans collected with incubation medium in both calorimeter cells (sample and reference cells) from sample scans. After normalization for molar concentration, a progress baseline was subtracted from experimental data. A non-two-state model was fitted to the molar excess heat capacity function of each sample, in order to estimate values of thermal transition temperature, calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy (ΔH_{vH}). The non-two-state model is the most convenient model to employ in analyses of differential scanning calorimetric data in cases where it is not known whether the transition is reversible or cooperative (A. Cooper, personal communication, October, 2005).

I. *Assessment of Peptidolytic Activity of Porcine Pepsin A*

Conventional structural methods usually furnish insights into changes involving the whole protein

molecule or, at least, major regions within the biomacromolecule. On the contrary, assessment of enzymatic activity has been deemed of utmost convenience for recognizing slight perturbations in conformation, taking place prior to the onset of global protein denaturation (Shukla, *et al.*, 2005; Ternström, *et al.*, 2005; Yoshimasu, *et al.*, 2004; Zoldák, *et al.*, 2003). In light of these considerations, peptidolytic activity of porcine pepsin A was assayed when it was considered valuable in complementing data on gross conformational changes.

I.1. *Aspects of Assay Design*

I.1.1. *Substrate*

Throughout the research conducive to the current dissertation, evaluation of the effect of denaturing agents on the peptidolytic activity of porcine pepsin A was accomplished resorting to the synthetic substrate H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe. This chromophoric hexapeptide was reported to be a good substrate for porcine pepsin A long ago (Martin, 1984; Salesse & Garnier, 1976), and it has been recurrently used for assaying pepsinolytic activity (Okoniewska, *et al.*, 1999; Okoniewska, *et al.*, 2000; Pletschke, *et al.*, 1995). The chromophoric hexapeptide is solely cleaved at the *p* – nitro – Phe – Nle peptide bond by porcine pepsin A (Martin, 1984), and as a result two tripeptides are formed: H – Leu – Ser – *p* – nitro – Phe (product A) and Nle – Ala – Leu – OMe (product B). The amino acid sequence of this synthetic peptide is similar to the primary structure of bovine *k*-casein, specifically on each side of the residues Phe105 and Met106 (Stewart, *et al.*, 1984). Cleavage of this bond is a key event in the milk-clotting chain, a process catalyzed by the enzyme under study (Drohse & Foltmann, 1989).

Even though natural substrates provide better perception of the enzyme behaviour *in vivo*, the employment of a small synthetic peptide as substrate brings some advantages. Herein, the most important aspect to consider is the likelihood for the experimental conditions (for instance, the presence of denaturing agents), in which the enzymatic activity is being assayed, to induce conformational changes on the proteinaceous substrate and, consequently, modify its proteolytic susceptibility. These changes would give rise to misleading experimental data and interpretations, when comparing information obtained in different experimental setups. Such drawback should be defeated or minimized by use of small synthetic peptides [with a sequence of amino acid residues analogous to that of a region of a natural substrate containing a peptide bond which fits the primary specificity of the enzyme (Kotler, *et al.*, 1988)]. In fact, it has been claimed that small peptide substrates (akin to the one used) usually display no stable or well-defined structure; instead, they exist as extended chains with random coil - like conformations (Dunn, 1989; Hamada & Goto, 2005). And, thus, structural factors (pertaining to the substrate) can be ruled out as being responsible for variations in the action of an enzyme under dissimilar experimental conditions and / or on a set of different peptide substrates.

I.1.2. *Temperature and pH*

Reproducible measurements of enzymatic activity call for controlled conditions of temperature and pH. Hydrolysis of the synthetic hexapeptide by porcine pepsin A was appraised at 25ºC and pH 3 – the same experimental conditions chosen for incubation periods preceding most assays throughout this investigation, in agreement to what was described in the section 2.E. '*Induction of Porcine Pepsin A Denaturation*'. pH 3 belongs to the interval of pH values within which pepsin A optimally catalyzes hydrolysis of the hexapeptide aforementioned.

I.2. *Standard Experimental Procedure*

I.2.1. *Preparation and Performance of Hydrolytic Reactions*

Each enzymatic reaction was preceded by a phase of substrate equilibration. An aliquot of a stock of the synthetic substrate H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe was dissolved in reaction medium composed of 0.05 M sodium acetate, 0.2 M sodium chloride and 4% (v/v) DMSO, titrated to pH 3, without or with a defined concentration of denaturant. The mixture was homogenized and incubated at 25ºC for 10 min. Each hydrolytic reaction was started by addition of enzyme. Depending on the aim of the assay, porcine pepsin A was previously incubated or not in a set of controlled experimental conditions. A protein aliquot was transferred into the reaction mixture, which was gently homogenized. The ratio of substrate to enzyme molar concentrations was typically $9.3*10^4$:1, so that steady-state levels of enzymatic activity could be measured before substrate depletion affected rates. Hydrolytic reactions were carried out in a total volume of 300 µL and at a constant temperature value of 25ºC. Each reaction was performed in duplicate. And, in general, each set of reactions was repeated at least twice.

Peptidolytic activity of porcine pepsin A was assessed through a discontinuous assay, by which reaction aliquots, withdrawn at timed intervals, were analysed by means of reversed phase - high performance liquid chromatography (HPLC). At four timed intervals within a total measurement period of 30 min, 60 µL - aliquots were collected from the reaction mixture, and vigorously mixed with 500 µL of 0.05 mM ammonium bicarbonate at pH 8. On exposing pepsin A to pH values beyond 7, it undergoes spontaneous structural disordering and instantaneous inactivation (Campos & Sancho, 2003; Lin, *et al.*, 1992b; Lin, *et al.*, 1993; Pletschke, *et al.*, 1995). Absence of measurable activity at pH 6.9 was confirmed. After addition of 40 µL of 1.5% (v/v) TFA, samples of the peptidolytic assay were stored at 4ºC or -20ºC, depending if they were to be analyzed immediately or within a few days, respectively. Absence of porcine pepsin A reactivation after addition of TFA was ascertained by appropriate control assays.

I.2.2. *Quantification of Hydrolytic Products*

Prior to reversed phase chromatographic analyses, samples were centrifuged at 14,000 rpm and 4ºC for 10 min. Per each sample, a 100 μL - aliquot of supernatant was injected onto a C18 reversed phase - HPLC column (*Kromasil 100* – 5μm – 25*0.4, a silica gel column purchased from *Teknokroma*), coupled to an HPLC system (*ÄKTA* from *Amersham Biosciences*). Automated chromatographs were operated and chromatograms were processed with the *Unicorn* software (*Amersham Biosciences)*. Samples were injected manually with a 250 μL *Hamilton* microsyringe, or by means of an automated sample injector, *Autosampler A-900* (*Amersham Biosciences*). Before application of each sample, the chromatographic column had been previously equilibrated with mobile phase composed of 70% (v/v) eluent A [0.1% (v/v) TFA in ultra-pure water] and 30% (v/v) eluent B [0.1% (v/v) TFA in acetonitrile]. Eluents were degassed with helium prior to use. Components of the reaction aliquots were eluted with a gradient of increasing concentrations of acetonitrile at a flow rate of 0.8mL/min. Details on each step of the elution method are given in the Table 3.1.

Table 3.1 – Elution method employed for separation of products and substrate in reaction aliquots by HPLC in a reversed phase C18 column, after quenching of porcine pepsin A - catalyzed hydrolysis of H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe.

Step Length (mL)	**Percentage of Eluent B** (%)
0.13	30
0.50	Sample Injection
2.77	30
8.29	Linear Gradient from 30 to 100
3.12	100
2.05	30

The chromophoric residue *p* – nitro – phenylalanine shows higher absorbance at 257 nm (Sarmento, 2002) and, thus, chromatographic runs were monitored by reading absorbance at this wavelength in order to detect product A. A typical chromatogram of a reversed phase - HPLC separation of components of a reaction aliquot is depicted in the Figure 3.1.

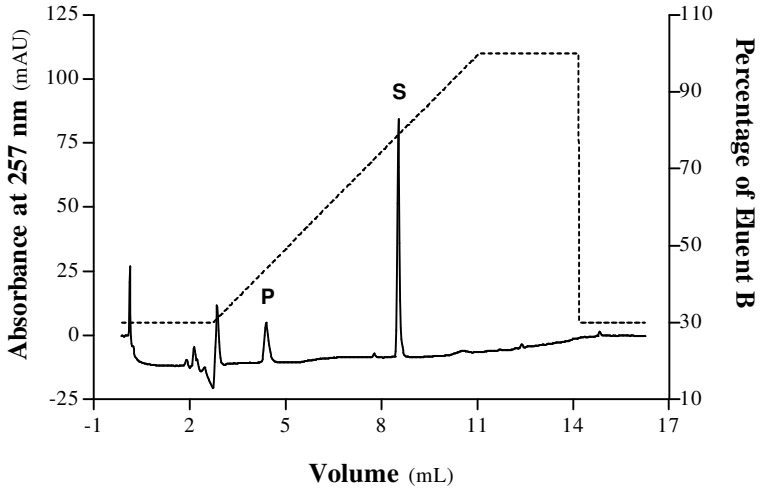

Figure 3.1 – Typical elution profile of reversed phase - HPLC separation of product A and substrate in reaction aliquots in a C18 column monitored at 257 nm, after quenching of porcine pepsin A - catalyzed hydrolysis of H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe. Sample was injected onto the column at 0 mL. **P** stands for H – Leu – Ser – *p* – nitro – Phe and **S** represents H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe. (___) Absorbance at 257 nm. (.....) Percentage of eluent B.

In the Figure 3.1, peaks labelled with **P** and **S** were assigned to product A and substrate, respectively, after mass spectrometric analyses. Absence of phenylalanine or *p* – nitro – phenylalanine residues in product B did not allow its identification by reading absorbance at 257 nm.

Peaks corresponding to the product A were integrated, and their areas converted into molar concentrations from calibration curves. Reaction progress curves were obtained by building graphs of the time-course of product formation, and their slopes were computed to give initial velocities.

J. *Solvent Accessible Surface Area Calculations for Porcine Pepsin A*

Prediction of the extent to which crucial residues are individually embedded in the three-dimensional structure of a protein could be of assistance in further rationalizing experimental data. By virtue of this, solvent accessibility of residues within natively folded porcine pepsin A was assessed by means of two freely available algorithms: (i) *GETAREA 1.1* provided by *Sealy Centre for Structural Biology* at the *University of Texas Medical Branch* and accessible at http://www.scsb.utmb.edu/cgi-bin/get_a_form.tcl (Fraczkiewicz & Braun, 1998); and (ii) *ASAView* available at http://www.netasa.org/asaview/ (Ahmad, *et al.*, 2004). Cartesian coordinates of atoms in the molecule of monoclinic porcine pepsin A were obtained from the *Protein Data Bank* (PDB), which can be accessed at http://www.rcsb.org/pdb/ and is now operated by the *Research Collaboratory for Structural Bioinformatics* (Berman, *et al.*, 2000). Upon submission of the PDB file identified as *4pep* (Sielecki, *et al.*, 1990), the two algorithms generated values of relative *solvent*

accessible surface area (SASA) for each residue. Expression of data as relative quantities legitimates comparisons between amino acid residues of dissimilar sizes.

Solvent accessible surface area of a molecule has been defined as the locus of the centre of a spherical probe (which generally is a solvent molecule), when it rolls on the van der Waals surface of the molecule without penetrating any of its atoms (Hayryan, *et al.*, 2005). Herein, an amino acid residue was deemed to be *exposed* if its relative solvent accessibility exceeded 50%; a residue displaying a relative solvent accessibility less than 20% was surmised to be *buried* (Fraczkiewicz & Braun, 1998). Whenever a residue was assigned a value of relative solvent accessible surface area falling in the 20 – 50% interval, it was surmised to be partially buried (or partially exposed).

CHAPTER IV

RESULTS & DISCUSSION

As emphasized in the chapter II '*Objectives & Outline'*, the main aspiration of the ongoing dissertation consisted in improving the knowledge on the conformational stability of native porcine pepsin A. Efforts in this direction involved engendering an approach by which the effects of selected denaturing agents (acetonitrile, guanidine hydrochloride and urea) on the natively folded state of porcine pepsin A were evaluated, with the purpose of furnishing some clues on the features accountable for preservation of its conformational integrity under extreme experimental conditions.

The present chapter concentrates on the experimental data acquired upon taking porcine pepsin A from native-like conditions to denaturing media and measurement of an array of properties. The investigation reported herein is based on integrated analyses of intrinsic spectral, hydrodynamic, calorimetric and enzymatic properties. Aside from description of results, brief interpretative remarks will be provided.

The strategy employed for surveying the dependence of conformational integrity of natively folded porcine pepsin A on the concentration of denaturants included the use of aqueous sodium acetate at pH 3 during incubation steps and assays. In keeping with the arguments presented in previous chapters, the demand for ensuring that the target of perturbation exhibited a natively ordered state laid beneath the choice of pH conditions. Evidence in favour of acquirement of a native conformational state by pepsin only at pH values below 3.5 has come from a recent report by Campos and Sancho (2003). Other details on the experimental conditions selected for induction of protein denaturation were addressed in the section 2.E. '*Induction of Porcine Pepsin A Denaturation*' of chapter III.

An inconvenience could arise from studying native porcine pepsin A. Being both a protein and a proteolytic enzyme, porcine pepsin A is capable of digesting itself. The autolytic nature of pepsin has long been noted. In a recent paper authored by Qiao and associates (2002), it was concluded that the higher the pH and pepsin concentration, the longer its half-life for peptide bond hydrolysis. In order to evaluate the extent of eventual self-proteolysis of porcine pepsin A and whether it would become or not an obstacle to an

accurate dissection of the effects of denaturants on the protein, electrophoretic analyses of the native protein were accomplished. As such, samples of porcine pepsin A were incubated at 2.857 μM in 10 mM sodium acetate at pH 3 and 25ºC. Incubation periods lasted no longer than 3 days. Selection of protein concentration during the incubation phase was limited by the maximum capacity of the gel wells and the minimum mass of protein that should be loaded to allow detection of autolysates. After preparation of samples according to the instructions given in the section 2.D. '*Assessment of Purity and Autolysis of Porcine Pepsin A*' of chapter III, polyacrylamide gel electrophoreses under denaturing and near-native conditions were performed. Protein bands were detected by silver nitrate staining.

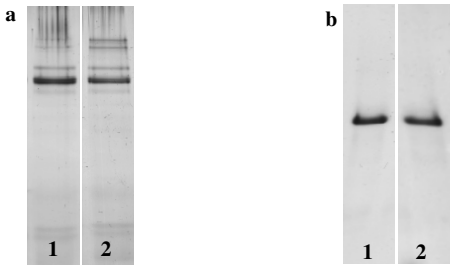

Figure 4.1 – Polyacrylamide gels resulting from vertical electrophoresis of samples of porcine pepsin A (**a**) in the presence and (**b**) in the absence of sodium dodecyl sulphate, after silver nitrate staining (the revelation step lasted 4 min and 45 sec). 0 and 3 days of incubation of porcine pepsin A at 2.857 μM in 10mM sodium acetate at pH 3 and 25ºC correspond respectively to lanes **1** and **2**.

Apart from a prominent band corresponding to porcine pepsin A, gel electrophoresis under denaturing conditions followed by silver nitrate staining revealed additional bands corresponding to molecular species with higher and lower molecular mass than the protein under investigation both at 0 and 3 days of incubation at pH 3 and 25ºC [see Figure 4.1(a)]. Contrarily, a single band was observed in each lane of a polyacrylamide gel resulting from vertical electrophoresis in the absence of sodium dodecyl sulphate of pepsin samples, after 0 and 3 days of incubation under the same conditions [see Figure 4.1(b)]. Technically, it should be reasonable to assume that extra bands observed in Figure 4.1(a) were due to the procedure employed for preparation of samples, which implied addition of detergents, chaotropes and reducing agents, as well as high temperature heating. Reduction of pepsin has been noted to take place with concomitant aggregation for long (Blumenfeld & Perlmann, 1961). And, hydrolysis of labile peptide bonds is well-known to be a recurrent consequence of exposure to high temperature and acidic pH (Volkin & Klibanov, 1991). Alternatively, the commercial preparation of porcine pepsin A could be heterogeneous, and bands detected below the band of intact protein could correspond to autodigestion products. Detection of such molecular species by gel electrophoresis under denaturing conditions and no detection by gel electrophoresis under near-native conditions are explicable if one assumes that these fragments were still held together in the protein, for instance, by disulphide bridges before submission to denaturing procedures. Either way, percentage of integer porcine pepsin A was estimated to be about 96.6% of the total amount of protein in the sample. This estimation was made from two sets of four samples of porcine pepsin A with varying masses of

protein, which were submitted to gel electrophoresis in the presence of sodium dodecyl sulphate and analysed by densitometry. In regard to occurrence of autolysis during experiments, it was, in principle, considered to be negligible, once no major differences were detected in electrophoretic profiles corresponding to samples submitted or not to incubation in the conditions specified above.

1. *ACETONITRILE-INDUCED DENATURATION OF PORCINE PEPSIN A*

Mechanisms by which organic solvents interfere with proteins are complex. Even though intense endeavours have been made to exploit non-aqueous enzymology and its practical applications (Arnold, 1990; Dordick, 1992; Gupta, 1992; Halling, 1994), the impact of organic solvents on protein conformations remains not thoroughly detailed. It is typically anticipated that the presence of organic solvents influences physicochemical properties of the medium and, consequently, hydration layers essential for biomacromolecule folding; thereby causing disruption and / or impacting the strength of electrostatic and van der Waals interactions, hydrogen bonds and the hydrophobic effect accountable for the maintenance of folded conformational states (Affleck, *et al.*, 1992; Gladilin & Levashov, 1998; Rehan & Younus, 2006; Revilla, *et al.*, 1986; Sirotkin, *et al.*, 2000; Thomas & Dill, 1993; Williams, *et al.*, 1995), or even inducing the formation of novel intramolecular interactions (Hamada & Goto, 2005). Direct binding of organic solvents to proteins has also been observed (Dennis, *et al.*, 2002; Fitzpatrick, *et al.*, 1993; Schmitke, *et al.*, 1998). From data on the effect of organic solvents on proteins, it should be possible to achieve a better understanding of the forces and features governing conformational stability, together with a clarification of the role of water in this scope.

As stated earlier, due to its unusual properties and the extensive knowledge accumulated over the years on its structure and function, porcine pepsin A may be considered a particularly good model for following such general problems as comprehending the determinants of conformational stability and the response of proteins to organic solvents. In regard to the latter issue, it should be important to emphasize that the natural environment of the protein under study is essentially aqueous. Pepsin A is an extracellular enzyme, mainly localized in the stomach lumen (Elliott & Elliott, 2001), and it is not used to be and work in non-aqueous milieus, in contrast to proteins associated to membranes, for instance. Porcine pepsin A can, thus, be employed as a suitable model for broadening the insight on the effective action of organic solvents on proteins, in alternative to the use of, for example, the well-known α-chymotrypsin and subtilisin (Griebenow & Klibanov, 1996; Kijima, *et al.*, 1996; Kreiner, *et al.*, 2005; Saborowski, *et al.*, 2004; Sato, *et al.*, 2000; Schmitke, *et al.*, 1997; Simon, *et al.*, 2001; Zaks & Klibanov, 1988).

Besides the individual nature of the protein and the water contents in the protein-solvent system (Gladilin & Levashov, 1998; Mattos & Ringe, 2001; Simon, *et al.*, 1998), the extent and type of perturbations in protein conformations triggered by organic solvents do also markedly depend on their own

physicochemical characteristics. Acetonitrile (CH_3CN) is an aprotic solvent (Elola & Ladanyi, 2005; Maroncelli, 1991; Olofsson, *et al.*, 2005), and it has an amphiphilic character, for it possesses both organic and polar functional groups (Dawson & Wallen, 2002). Subsequently, it is less hydrophobic (Maroncelli, 1991) than other typical organic solvents like, for example, toluene or benzene. Indeed, acetonitrile is completely water-soluble throughout the mole fraction range, and the base-10 logarithm of its octanol / water partition coefficient, log *P*, is relatively low (-0.34) (Khmelnitsky, *et al.*, 1991b). A number of authors have seemingly agreed in a microheterogeneous behaviour for water-acetonitrile mixtures (Bertie & Lan, 1997; Catalán, *et al.*, 2003; Dawson & Wallen, 2002; Marcus & Migron, 1991; Mountain, 1999; Takamuku, *et al.*, 1998; Venables & Schmuttenmaer, 1998). However, the nature of intercomponent interactions and water structural changes induced by addition of this organic solvent remain controversial questions at this writing.

Acetonitrile has found diverse applications in many branches of chemistry. In protein science, acetonitrile has gained practical significance through recurrent use as an organic modifier in purification of peptides and protein fragments by reversed phase - HPLC (Gekko, *et al.*, 1998; Kromidas, 2000; Syed, 1992), and as a component of reaction mixtures for peptide synthesis catalyzed by several proteinases (Bordusa, 2002; Cerovský, 1990; Fischer, *et al.*, 1991; Noritomi, *et al.*, 1995), including pepsin (Bemquerer, *et al.*, 1994).

Alongside the description and interpretation of the experimental data on acetonitrile-induced denaturation of porcine pepsin A, a brief characterization of the protein conformational state under native conditions will be provided in the forthcoming sections. Collection of most data described in the following sections was typically preceded by an incubation step. Unless otherwise stated, the protein was submitted to increasing concentrations of organic solvent for 1 h at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used for preparation of binary aqueous mixtures of acetonitrile and as reference medium. It is worth noting that, even though composition of binary mixtures is usually expressed in terms of mole or volume fraction, it was decided to use molarity with the aim of facilitating comparative analyses with experimental data obtained on porcine pepsin A in the presence of chaotropes.

A. *Structural Properties*

A.1. *Secondary Structure*

Far-UV circular dichroic spectra of porcine pepsin A in 10 mM sodium acetate at pH 3 corresponded to a well-folded conformational state. These traces were characterized by a prominent negative band with two maxima of similar magnitude at about 208 and 216 nm, in addition to a positive band with a single maximum at around 193 nm (see Figure 4.2). A peak centred at 216 nm is a distinctive feature in circular dichroic spectra of β-sheets (Rodger & Ismail, 2000). Thus, circular dichroic absorbance at this wavelength was taken to be the best indicator for alterations in β-structure contents and, in principle, in the

overall secondary structure of porcine pepsin A. The signal at 222 nm, which typically becomes a minimum in far-UV circular dichroic spectra of proteins rich in α-helices, was overlapped by the dominating peak at 216 nm. This observation is compatible with a predominance of β-sheets in the secondary structure of natively folded porcine pepsin A (Andreeva, *et al.*, 1984). Therefore, variations in helicity were monitored as changes in circular dichroic signal at 208 nm.

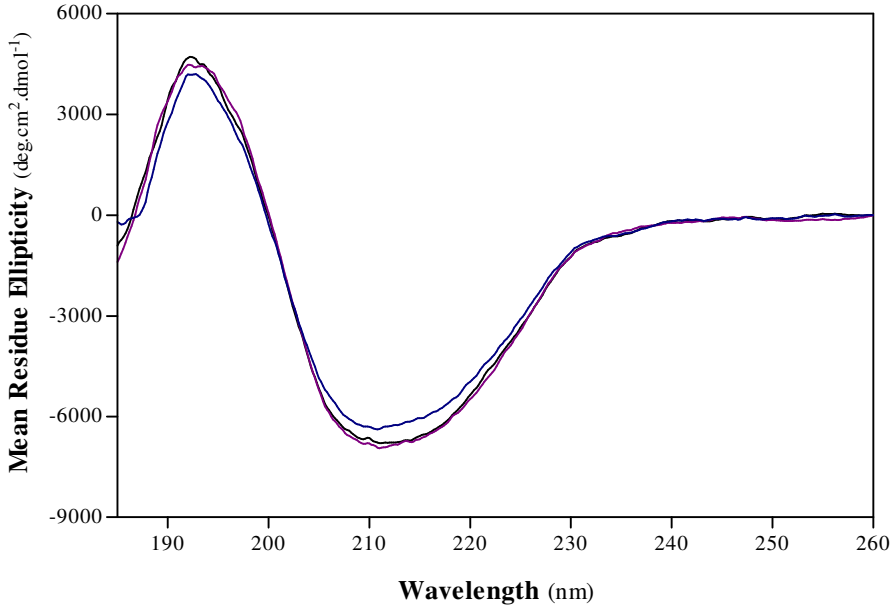

Figure 4.2 – Far-UV circular dichroic spectra of porcine pepsin A collected after incubation in 10 mM sodium acetate at pH 3 and 25 ºC during 0 h (___), 1 h (___) and 24 h (___).

Circular dichroic spectra of porcine pepsin A in the amide region were measured after incubation during 0, 1 and 24 h in aqueous medium at pH 3 and 25ºC. Superposition of spectra corresponding to 0 and 1 h (see Figure 4.2) indicated that the protein under study remained natively folded during 1 h - incubation. Numerical estimation of contents of secondary structural units from deconvolution of far-UV circular dichroic spectra yielded 10% helices and 42% strands. Such data are in fair agreement with information deposited in the *Protein Data Bank* (Berman, *et al.*, 2000) for the crystal structure [PDB code *4pep* (Sielecki, *et al.*, 1990)]. Subsequent to a 24 h-incubation, there was a slight reduction in the absolute value of circular dichroic signal by porcine pepsin A, which reflected a decrease in helices and strands contents of about 0.3% and 1.3%, respectively.

With the purpose of studying the effect of acetonitrile on regular elements of secondary structure of porcine pepsin A, far-UV circular dichroic spectra were collected, after exposure of the target protein to growing concentrations of the organic solvent for 1 h at 25ºC.

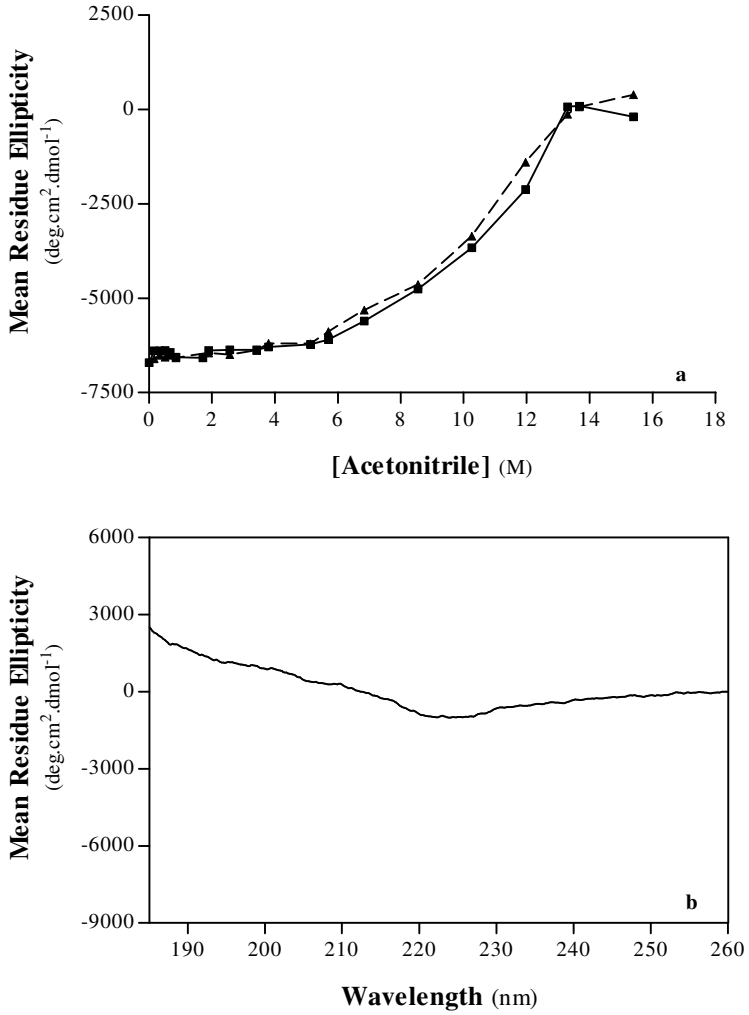

Figure 4.3 – **(a)** Dependence of the mean residue ellipticity of porcine pepsin A at 208 nm (triangles; _ _) and 216 nm (squares; ___) on acetonitrile concentration. Data were obtained from far-UV circular dichroic spectra, which were recorded after 1 h of exposure of porcine pepsin A to increasing concentrations of organic solvent at 25ºC. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. **(b)** Far-UV circular dichroic spectrum of porcine pepsin A collected after 1 h - incubation at 25ºC in 15.39 M acetonitrile.

As can be seen in Figure 4.3(a), a raise in the acetonitrile concentration in the organic-aqueous solvent system up to 5.13 M did not cause striking modifications in the contents of β-sheets and α-helices. On the other hand, concentrations equal to and greater than 5.70 M seemed to induce a gradual disarray in the secondary structure; so that, at and above 13.30 M of organic solvent in the system, mean residue ellipticity values close to zero indicated the attainment of an unfolded state of porcine pepsin A. The spectrum shown in the lower panel in the Figure 4.3 was recorded for the protein under investigation in 15.39 M acetonitrile, and it is consistent with a conformational state devoid of regular secondary structural elements.

Even though the dependence of ordered structure on the acetonitrile concentration was solely reported by circular dichroic absorbance at 208 and 216 nm in Figure 4.3(a), an increase in the concentration of organic solvent to values equal to and higher than 5.70 M resulted in a general decrease in absolute values

of the signal at every wavelength between 185 and 230 nm. It is worth mentioning, however, the existence of an isodichroic point at about 200 nm, as it shall be demonstrated later.

Closeness of curves in the Figure 4.3(a) indicates that decay of secondary structural elements was concurrent. Moreover, such acetonitrile-induced far-UV circular dichroism transitions follow sigmoidal curves. On account of such observation and the existence of an isodichroic point, it is claimed that, under the selected experimental conditions, loss of β-sheets and α-helices in porcine pepsin A promoted by acetonitrile occurred as a single-step transition.

Finally, the dependence of acetonitrile-provoked changes in the secondary structure of porcine pepsin A upon the length of incubation step was investigated. The general perception from Figure 4.4(a) was that distinct time profiles of the variation of circular dichroic absorbance at 216 nm could be observed for concentrations of organic solvent belonging to different regions of the conformational transition depicted in the Figure 4.3(a). Furthermore, each set of spectra generated after 0, 2 and 24 h of exposure to organic solvent conserved an isodichroic point at around 200 nm [see Figure 4.4(b, c, d)]. In principle, this observation indicates that the disappearance of secondary structural elements followed a single-step mechanism irrespective of the period of incubation (in the range from 0 to 24 h) in increasing concentrations of acetonitrile.

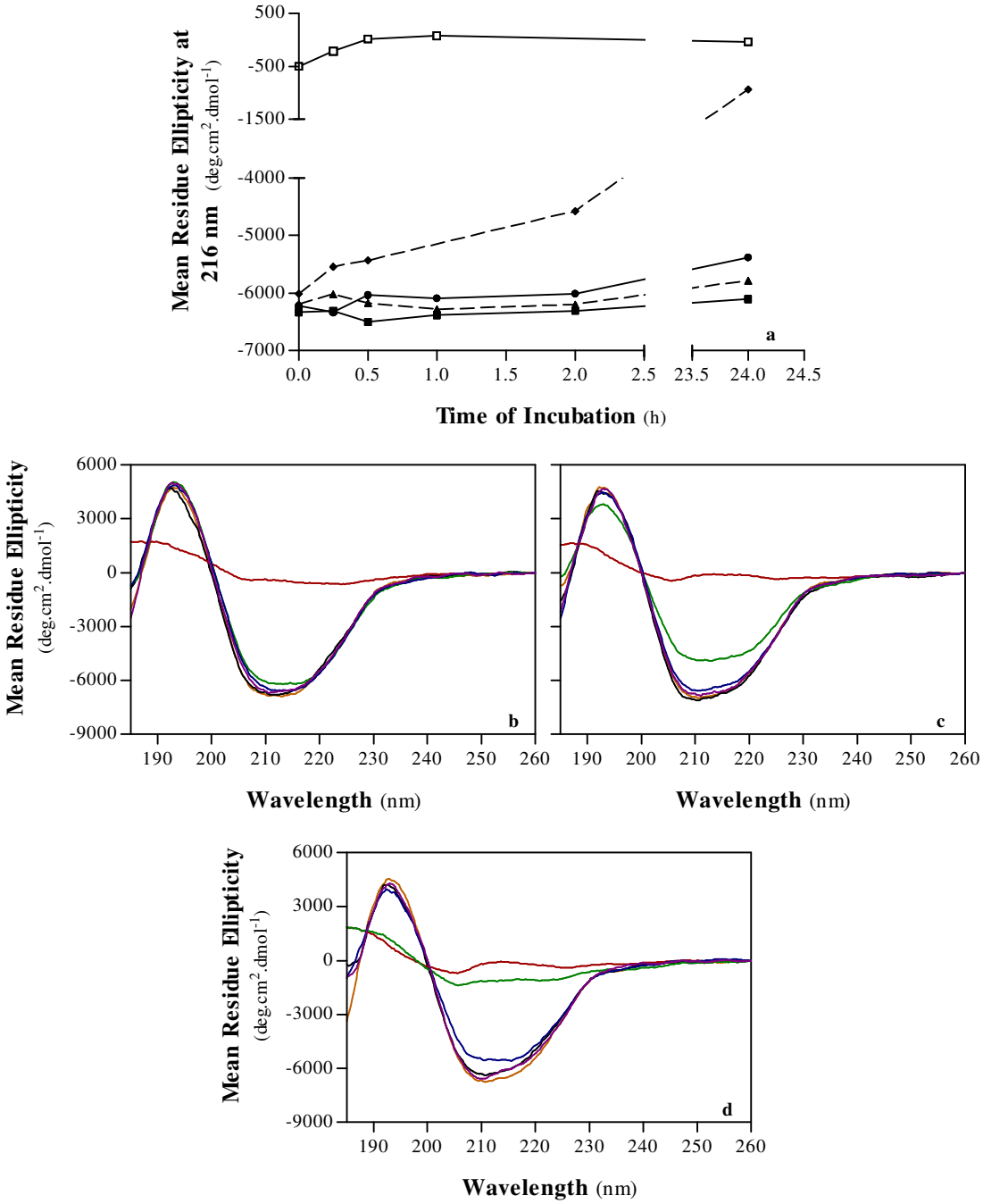

Figure 4.4 – Time-dependence of acetonitrile-induced changes in pepsin secondary structure. Data were obtained from far-UV circular dichroic spectra, which were collected after exposure of porcine pepsin A to selected concentrations of organic solvent at 25ºC during defined periods of time. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. **(a)** Mean residue ellipticity at 216 nm as a function of length of incubation in 1.90 M (filled squares; ___), 3.80 M (filled triangles; _ _), 5.70 M (filled circles; ___), 9.50 M (filled diamonds; _ _), and 13.30 M (empty squares; ___) acetonitrile. **(b)** Far-UV circular dichroic spectra of porcine pepsin A in aqueous medium (___), and in 1.90 M (___), 3.80 M (___), 5.70 M (___), 9.50 M (___), and 13.30 M (___) acetonitrile without a previous incubation step. **(c)** Far-UV circular dichroic spectra collected after a 2 h - incubation in aqueous medium (___), in 1.90 M (___), 3.80 M (___), 5.70 M (___), and 9.50 M (___) acetonitrile, and after a 1 h - incubation in 13.30 M acetonitrile (___). **(d)** Far-UV circular dichroic spectra collected after a 24 h - incubation in aqueous medium (___), and in 1.90 M (___), 3.80 M (___), 5.70 M (___), 9.50 M (___), and 13.30 M (___) acetonitrile.

In the absence of an incubation phase, far-UV circular dichroic spectra of porcine pepsin A in an organic-aqueous solvent system containing from 1.90 to 9.50 M acetonitrile were roughly similar to the spectrum of native protein [see Figure 4.4(b)]. On the other hand, 13.30 M of organic solvent triggered an instantaneous and marked defeat in the contents of structural units supportive of pepsin secondary structure, as judged by the corresponding featureless spectrum.

As seen in Figure 4.4(a), incubation of porcine pepsin A at low and intermediary concentrations of acetonitrile (1.90, 3.80 and 5.70 M) during periods shorter than 1 h resulted in a trendless variation of the spectroscopic signal within a narrow interval of values. While a slight reduction in ordered structural elements could be observed from 2 to 24 h of incubation at 3.80 and 5.70 M of organic solvent (concentration values close to the highest bound of the pre-transition region), equilibrium was nearly reached when exposure to 1.90 M of organic solvent lasted longer than 1 h.

In respect of protein-solvent systems containing 9.50 M acetonitrile [concentration value near the midpoint concentrations of the transitions depicted in the Figure 4.3(a)], there were glaring alterations in the mean residue ellipticity value at 216 nm as the length of the incubation period varied. Secondary structure was increasingly disrupted, so that upon a 2 h - incubation, the far-UV circular dichroic spectrum at 9.50 M acetonitrile was remarkably different from those corresponding to lower concentrations of organic solvent [see Figure 4.4(c)]. And, even though spectra generated after a 24 h - incubation corresponded to a seriously disordered conformational state, structural damages were not as pronounced as those observed at 13.30 M acetonitrile without previous incubation [see Figure 4.4(d)].

As stated earlier, acetonitrile at 13.30 M was found to exert an immediate denaturing action on porcine pepsin A [see Figure 4.4(a)]. Conformational perturbation progressed towards disappearance of the ordered arrangement as incubation periods were extended from 0 to 1 h. Thereafter, equilibrium was practically attained.

A.2. *Tertiary Structure*

Conformational modifications in porcine pepsin A occurring as a result of changes in acetonitrile concentration were additionally surveyed by measuring circular dichroic absorbance in the near-UV region. It is well-established that near-UV circular dichroism is responsive to minor changes in the integrity of rigid tertiary structures, for it reflects surrounding environments and relative orientations of side chains of aromatic amino acid residues in proteins (Kang, *et al.*, 1994a; Schmid, 1990). Notwithstanding details on tertiary structural features cannot be directly obtained by appraisal of near-UV circular dichroic spectra, great applicability has been found in following disordering transitions (Woody, 1994), and comparing between different conformational states (Kelly & Price, 2000; Rodger & Ismail, 2000; Schmid, 2005).

As illustrated in Figure 4.5, the near-UV circular dichroic spectrum of porcine pepsin A in native-like conditions (10 mM sodium acetate at pH 3) was characterized by positive bands in the wavelength range from 260 to 300 nm, with a fine peak at 290 – 292 nm. A peak close to 290 nm is characteristic of

contributions from tryptophan residues.

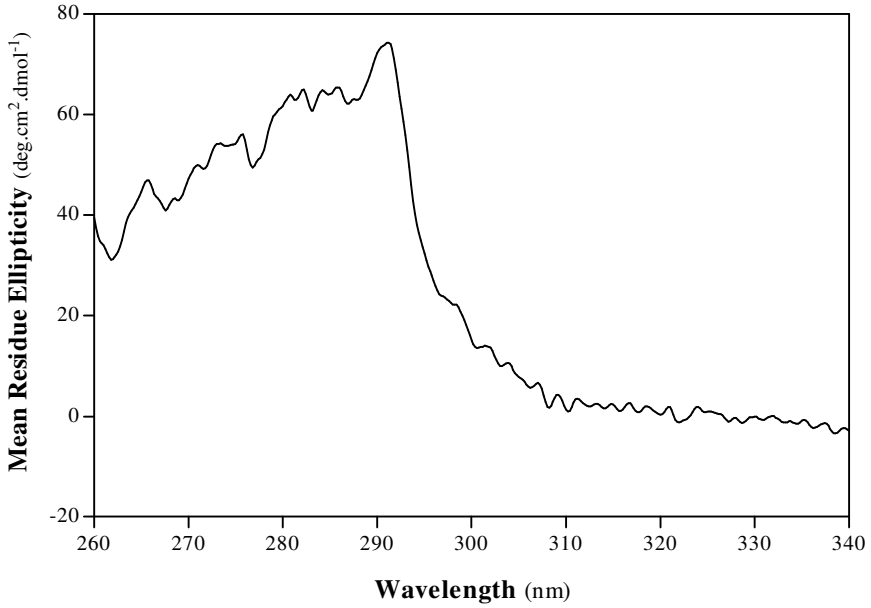

Figure 4.5 – Near-UV circular dichroic spectrum of porcine pepsin A collected after incubation in 10 mM sodium acetate at pH 3 and 25 ºC during 1 h.

Although the protein under investigation contains fourteen phenylalanine, sixteen tyrosine and five tryptophan residues, and a characteristic wavelength profile has been assigned to each of the aromatic amino acids (Kelly & Price, 2000; Woody, 1994), circular dichroic absorbance at 290 nm was elected to report changes in tertiary structure, in detriment of other wavelengths, because: (i) porcine pepsin A owns three disulphides, and spectral ranges associated with contributions of phenylalanine residues and weak transitions of disulphide groups are typically overlaid; (ii) even though the number of tyrosine residues (partially buried and exposed) is higher than that of tryptophan residues, circular dichroic absorbance by the former may be hidden by contributions of the latter, especially, at longer wavelengths.

In the Figure 4.6(a), mean residue ellipticity at 290 nm was plotted against the molar concentration of acetonitrile in the solvent system. Data were obtained after submission of porcine pepsin A to a 1 h - incubation at 25ºC in different solvent conditions.

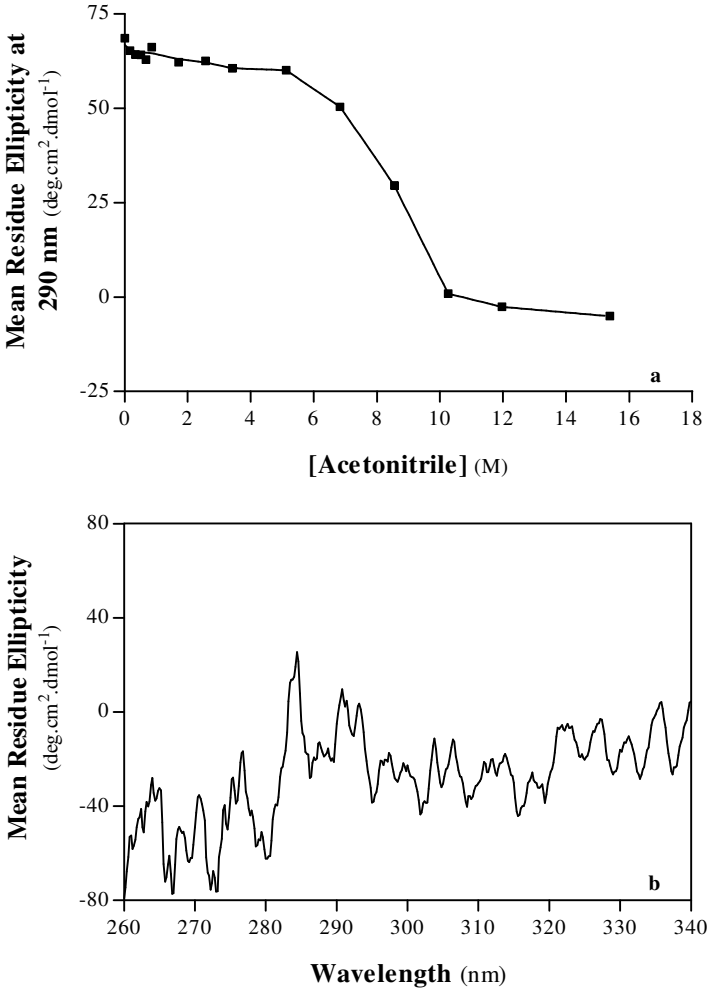

Figure 4.6 – **(a)** Dependence of the mean residue ellipticity of porcine pepsin A at 290 nm on acetonitrile concentration. Data were obtained from near-UV circular dichroic spectra, which were recorded after 1 h of exposure of porcine pepsin A to increasing concentrations of organic solvent at 25ºC. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. **(b)** Near-UV circular dichroic spectrum of porcine pepsin A collected after 1 h - incubation at 25ºC in 15.39 M acetonitrile.

Concurrently with experimental evidence obtained from circular dichroism in the deep-UV region, concentrations of organic solvent up to approximately 5.13 M did not exert a marked effect on the tertiary structure of porcine pepsin A [see Figure 4.6(a)]. However, an overall decreasing trend of aromatic band intensities in the wavelength range from 260 to 300 nm could be observed up to around 10.26 M acetonitrile, which hinted at an increase in mobility of aromatic side chains. Hence, at concentrations higher than 5.13 M of organic solvent, porcine pepsin A became progressively less structured. At acetonitrile concentrations equal to and higher than 11.97 M, the ordered arrangement of aromatic amino acid residues was largely lost. Indeed, near-UV circular dichroic spectra at high acetonitrile concentrations showed very small negative ellipticity values and were devoid of typical aromatic bands, which would be consistent with a highly unfolded conformational state [see Figure 4.6(b)]. In view of the sigmoidal shape and relative sharpness of the transition curve in the Figure 4.6(a), destruction of the tertiary structure of porcine pepsin A conformed to

an essentially cooperative, single-step process.

The near-UV circular dichroic spectrum of porcine pepsin A generated upon exposure to 15.39 M acetonitrile shown in the Figure 4.6(b) further conveyed aggregation of denatured conformations. Ultraviolet absorption spectra of porcine pepsin A in the presence of growing concentrations of organic solvent did also evidence the occurrence of aggregation. Samples were prepared and incubated in experimental conditions identical to those set for samples destined to be analyzed by circular dichroism, and ultraviolet absorption spectra were recorded according to the guidelines provided in the subsection 2.C.1.'*Ultraviolet Absorption Spectroscopy*' of chapter III.

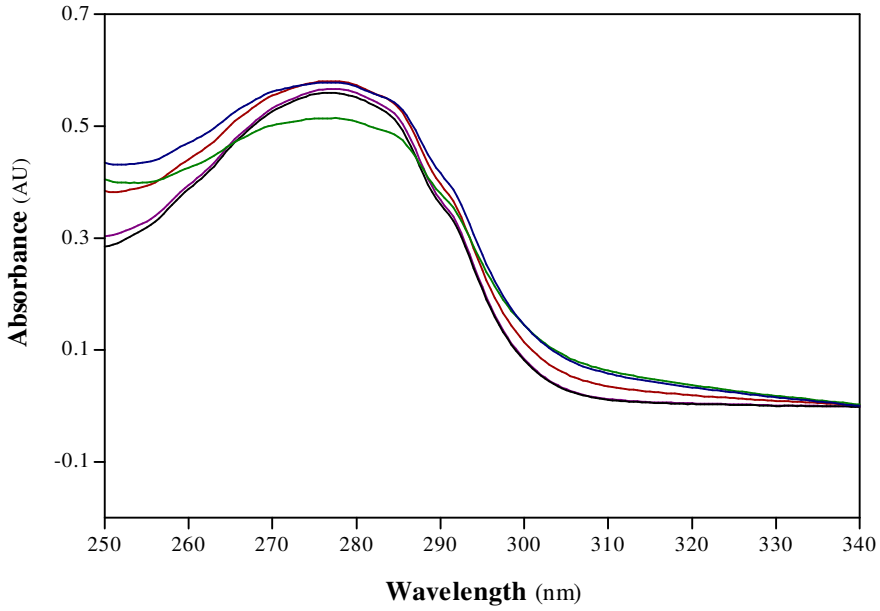

Figure 4.7 – UV absorption spectra of porcine pepsin A recorded after 1 h - incubation at 25ºC in aqueous medium (___), and 1.90 M (___), 3.80 M (___), 5.70 M (___) and 7.60 M (___) acetonitrile. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3.

In samples at about 14.286 μM of porcine pepsin A, aggregates in suspension were visibly detected at concentrations of organic solvent equal to and higher than 5.70 M. Nonetheless, a sloping baseline above 310 nm was observed in ultraviolet absorbance spectra recorded in the presence of acetonitrile concentrations as low as 3.80 M (see Figure 4.7).

Modifications in tertiary structure of porcine pepsin A promoted by an increase in acetonitrile concentration were fatherly probed by performing intrinsic fluorescence measurements in the microenvironment of tryptophan residues. Indeed, intrinsic fluorescence spectroscopy is a tool suitable for investigating modifications in solvent shielding and polarity of the microenvironment of aromatic amino acid residues (Eftink, 1994; Eftink & Maity, 2000; Kijima, *et al.*, 1996; Ramsay & Eftink, 1994; Roder, *et al.*, 2005), thereby providing information on conformational changes complementary to those retrieved from circular dichroism in the near-UV region.

Before excitation at 295 nm and recording of fluorescence emission spectra, porcine pepsin A was exposed to increasing concentrations of acetonitrile during 1 h at 25ºC. Spectra were examined in terms of shifts in the wavelength of maximum fluorescence emission. Such parameter is more convenient for tracking changes in the molecular environment of tryptophan residues resulting from conformational alterations in local or proximal areas in detriment of fluorescence intensity. While curves of wavelength of maximum fluorescence emission by proteins are amenable to straightforward interpretations, as it shall be seen later; variations in tryptophanyl fluorescence intensity are less informative in terms of the degree of solvent exposure of fluorophoric groups, for it may either increase or decrease upon protein unfolding (Schmid, 1990; Schmid, 2005).

Two comments emerge from fluorescence emission spectra of porcine pepsin A generated in the absence of organic solvent and presented in the Figure 4.8. Firstly, the tertiary structure of porcine pepsin A seems to remain practically intact on incubation during 1 h in aqueous medium at pH 3, as evidenced by the marked overlapping of fluorescence emission spectra recorded with or without a previous incubation step.

Secondly, fluorescence emission spectra of porcine pepsin A under native-like conditions showed a maximum at around 347.2 nm, which is typical of a protein with partially buried tryptophan residues (Mazzini, *et al.*, 2002; Parisi, *et al.*, 2003).

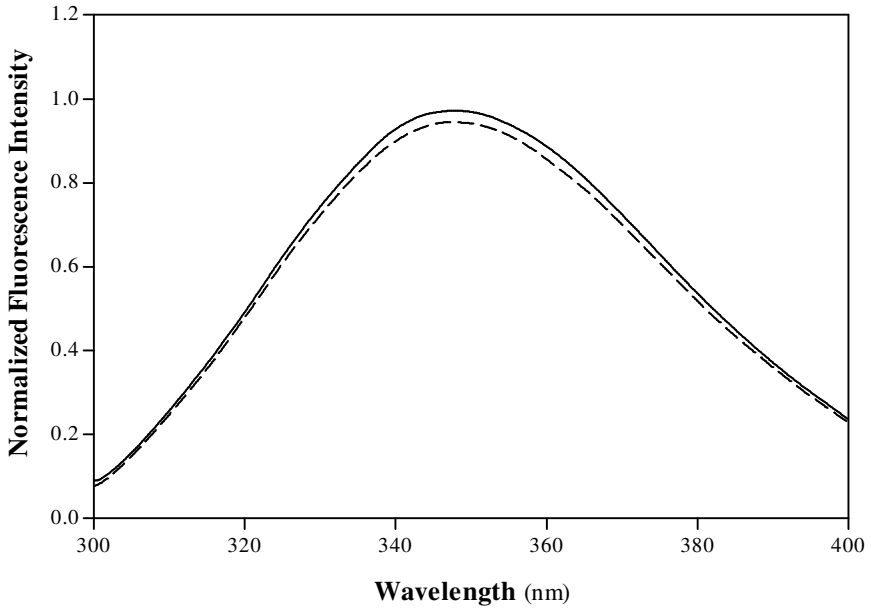

Figure 4.8 – Intrinsic tryptophanyl fluorescence emission spectra recorded after incubation of porcine pepsin A during 0 h (___) and 1 h (_ _) at 25ºC in sodium acetate at pH 3.

Fluorescence of a tryptophan residue is largely determined by its solvent accessibility (Guzzi, *et al.*, 1999). As such, estimation of the extent to which tryptophan residues are individually embedded in the protein structure was performed in order to rationalize fluorescence properties of natively folded porcine pepsin A. Relative solvent accessible surface areas were assessed for each residue in the crystal structure of

porcine pepsin A (PDB code *4pep*). Values yielded by two different softwares (*GETAREA 1.1* and *ASAView*) were consensual (see Table 4.1).

Table 4.1 – Values of relative solvent accessible surface area assigned to tryptophan residues in natively folded porcine pepsin A by the algorithms *GETAREA 1.1* and *ASAView*.

	Relative Solvent Accessible Surface Area (%)				
	Trp39	**Trp141**	**Trp181**	**Trp190**	**Trp299**
GETAREA 1.1	0.4	43.0	29.8	0	2.3
ASAView	1.2	41.6	34.5	0	3.3

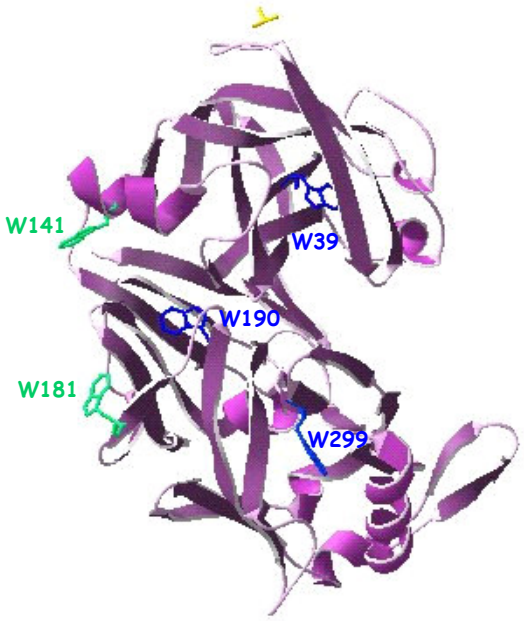

Figure 4.9 – Distribution of tryptophan residues in the three-dimensional structure of porcine pepsin A (PDB code *4pep*). The main chain trace is shown in cartoon format, and the phosphoryl group is coloured in yellow. Tryptophan residues are coloured according to their accessibility: buried residues are shown in blue and partially buried residues are shown in green. This figure was generated by means of the interface *Swiss-PdbViewer 3.7*.

According to the Table 4.1 and as depicted in the Figure 4.9, the five tryptophan residues in natively folded porcine pepsin A are differently accessible to solvent. It follows that their contributions to the overall fluorescent signal observed for the protein must be dissimilar. On one hand, Trp39, Trp190, and Trp299 are extremely shielded against solvent, once they are well-buried in the protein three-dimensional structure. Inclusively, the second residue mentioned was estimated to be completely inaccessible. On the other hand, an intermediate status of exposure was assigned to Trp141 and Trp181. Almost certainly, bulk intrinsic tryptophanyl fluorescence emission by natively folded porcine pepsin A mainly originates from the partially buried residues – Trp141 and Trp181.

It has been claimed that light emission by exposed fluorophores is dependent on properties of the solvent system, namely, polarity (Kijima, *et al.*, 1996) and viscosity (Winkler, 1967). These effects must be considered, when exploring fluorescence properties of a protein in solvent systems with different compositions. As such, fluorescence spectra were acquired for a tryptophan analogue completely exposed to solvent, the N-acetyl-L-tryptophanamide (NATA), submitted to identical experimental conditions set for fluorescence measurements of protein samples, in order to gauge specific effects of solvent composition on porcine pepsin A.

In Figure 4.10(a), wavelengths of maximum fluorescence emission by porcine pepsin A and NATA were plotted against acetonitrile contents in the protein-solvent system. To cope with the direct influence of solvent polarity on intrinsic tryptophanyl fluorescence emission by porcine pepsin A, the parameter $\Delta\lambda_{max}$ was computed and plotted as well [see Figure 4.10(b)]. $\Delta\lambda_{max}$ is defined as the difference between the two emission maxima of NATA and porcine pepsin A (Kijima, *et al.*, 1996). If $\Delta\lambda_{max}$ is close to zero, the environmental polarity of tryptophan residues in a protein is surmised to be tantamount to that of the tryptophan model compound. In other words, this should be indicative of tryptophan residues fully exposed to the solvent as a result of protein unfolding.

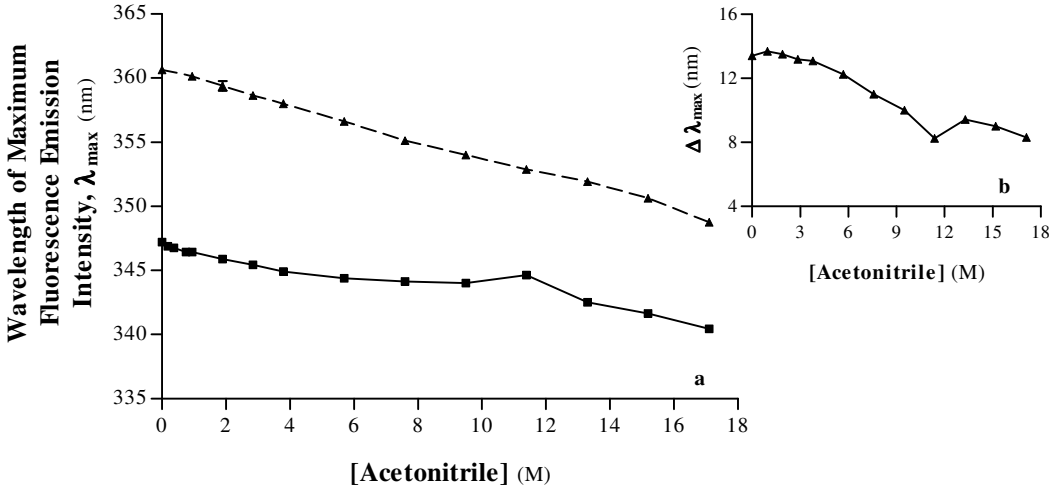

Figure 4.10 – **(a)** Changes in the wavelength of maximum fluorescence emission by NATA (triangles; _ _) and porcine pepsin A (squares; ___) induced by acetonitrile. **(b)** Effect of increasing concentrations of acetonitrile on the difference between the wavelengths of maximum fluorescence emission by NATA and porcine pepsin A. Data were taken from intrinsic tryptophanyl fluorescence emission spectra, which were collected after 1 h of exposure of NATA and porcine pepsin A to increasing concentrations of organic solvent at 25ºC. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot.

For binary aqueous-organic mixtures, the position of fluorescence emission spectra of NATA steadily moved to shorter wavelengths over the entire range of concentrations of acetonitrile spanned [Figure 4.10(a)], as a result of being surrounded by a medium of decreasing polarity. Solvent rearrangements during the lifetime of the excited state of the residue induced a decrease in its stabilization. An increase in the energy released by fluorophoric groups in the tryptophan model compound to the media accounts for the observed blue shift.

Distinct regions may be identified in the curve representing the variation in the wavelength of maximum fluorescence emission by porcine pepsin A with increasing concentrations of acetonitrile. On raising the concentration of organic solvent from 0 to approximately 5.70 M, fluorescence emission spectra underwent a blue shift, due to an increase in the hydrophobicity of the surrounding medium. Such spectral behaviour is comparable to that observed for the model. This is taken to mean that the tertiary structure of porcine pepsin A did not suffer noticeable disarrangements within the mentioned range of acetonitrile concentrations; or, at least, acetontrile-induced conformational changes did not occur in areas proximal to tryptophan residues. Such conclusion is in agreement with the plot in the Figure 4.10(b), according to which $\Delta\lambda_{max}$ suffered only a small shift of approximately 1.16 nm on going from 0 to 5.70 M acetonitrile.

From 5.70 to about 11.40 M acetonitrile, the position of fluorescence emission spectra of porcine pepsin A remained essentially invariable (average values changed from 344.38 to 344.63), which may result from the cancellation of, at least, two opposite effects. On one hand, tryptophan residues were becoming more exposed in response to the perturbation induced by increasing contents of acetonitrile in the solvent system. This would lead to a red shift in the emission maximum. Simultaneously, the wavelength of maximum fluorescence emission would diminish owing to a decrease in the polarity of the surrounding media, as demonstrated by the spectral behaviour of the tryptophan model compound [see Figure 4.10(a)]. This explanation is supported by the increasing closeness between the wavelengths of maximum emission of NATA and porcine pepsin A in this range of acetonitrile concentrations. In all likelihood, tryptophan residues of porcine pepsin A in an acetonitrile-aqueous solvent system attained their highest level of exposure at around 11.40 M of organic solvent, and disorder in tertiary structure should not significantly proceed above this concentration.

In the transition from 11.40 to 13.30 M acetonitrile, variation in $\Delta\lambda_{max}$ as a function of acetonitrile concentration underwent a clear break [see Figure 4.10(b)]. Pronounced aggregation of denatured conformations may rationalize a slight increase in $\Delta\lambda_{max}$. Above 13.30 M acetonitrile, the observed blue shift could solely arise from increasing hydrophobicity of the surrounding media. Environmental hydrophobicity should influence those tryptophan residues in the final denatured conformational state which were more accessible to the solvent than in the native conformational state, albeit not yet fully exposed.

Acetonitrile-mediated conformational transition in porcine pepsin A tracked by intrinsic tryptophanyl fluorescence spectroscopy described above was in good agreement with the data obtained from the near-UV circular dichroic spectra. However, fluorescence measurements did not point to a complete exposure of the tryptophan residues to the solvent, which might be expected for the unfolded denatured state indicated by the circular dichroism data. At 11.40 M acetonitrile, $\Delta\lambda_{max}$ was approximately equal to 8.25 nm, which is suggestive of not fully exposed tryptophan residues in denatured conformations. This finding may be explained by both aggregative effects and persistence of local structure at high concentrations of organic solvent. Porcine pepsin A contains three disulphides which impede attainment of random coil - like conformations by imposing topological constraints and, hence, hamper full exposure of tryptophan residues known to be highly buried in the native conformational state. In fact, Trp39 is a close neighbour of the disulphide bridge established between Cys45 and Cys50 both in the backbone and in the three-dimensional structure, as illustrated in Figure 4.11. In addition, disulphide-bonded Cys206 / Cys210 and Cys249 / Cys282

are spatially proximal to the Trp299.

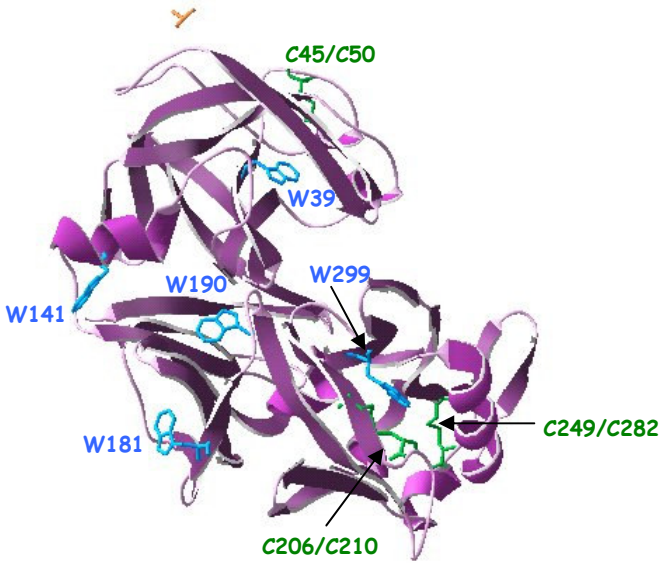

Figure 4.11 – Distribution of tryptophan and cysteine residues in the three-dimensional structure of porcine pepsin A (PDB code *4pep*). The main chain trace is shown in cartoon format, and the phosphoryl group is coloured in orange. Tryptophan residues are coloured in blue. Cysteine residues involved in disulphide bridges are coloured in green. This figure was generated by means of the interface *Swiss-PdbViewer 3.7*.

A.3. *Interpretative Remarks*

Experimental data previously described in the ongoing section evidence that, on incubation of porcine pepsin A in growing concentrations of acetonitrile during 1 h at 25ºC, significant spectral changes were observed above 5.13 – 5.70 M of organic solvent [see Figures 4.3(a), 4.6(a) and 4.10]. Separate evaluation of different structural properties suggests that destruction of secondary and tertiary structures occurred as monophasic transitions. However, a comparative analysis reveals that the tertiary arrangement was fully lost before the secondary structure. In fact, when followed by circular dichroic absorbance at 208 and 216 nm, the structured-to-unstructured transition of porcine pepsin A had midpoint concentrations of 10.69 ± 0.248 M and 10.79 ± 0.345 M, respectively. On the other hand, the disordering transition measured by near-UV circular dichroism afforded a midpoint concentration of 8.42 ± 0.142 M. Due to non-coincidence of denaturation transitions determined by different spectroscopic probes (*i. e.*, transitions occurred within different ranges and were centered at different concentration values; see Figure 4.12), it may well be that, in the selected experimental setup, acetonitrile-promoted unfolding of porcine pepsin A conformed to a multi-state model.

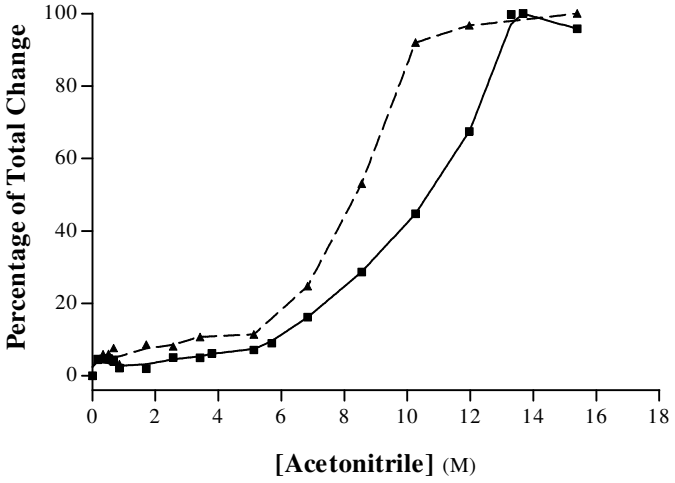

Figure 4.12 – Changes in structural properties of porcine pepsin A upon exposure to growing concentrations of acetonitrile for 1 h and 25ºC. Data are represented as percentage of the total change in the mean residue ellipticity of porcine pepsin A at 216 nm (squares; ___) and 290 nm (triangles; _ _) occurring over the range of denaturant concentration spanned.

Ascertainment of occurrence of one or more thermodynamically stable and populous intermediary conformational states, in the transition between natively folded and unfolded macrostates, demands equilibrium measurements. In reality, a 1 h - incubation was not enough for attaining denaturation equilibrium in all acetonitrile concentrations within the range spanned, as inferred from data on the kinetics of secondary structural changes occurring within porcine pepsin A in aqueous-organic mixed solvents [see Figure 4.4(a)]. Equilibrium is reached more slowly in samples with acetonitrile concentrations in the transition region of the curve in the Figure 4.3(a), which is a common observation in protein denaturation (Pace, *et al.*, 2005). In respect of the end-point of acetonitrile-assisted unfolding of the protein under investigation, it consisted of a structureless conformational state, albeit not exactly random coil - like, with appreciable intermolecular association. It should be stressed that the unfolding reaction was early accompanied by aggregative events, which could be detected even before the onset of global conformational changes. Besides, protein precipitation was noted in binary aqueous-organic mixtures with high acetonitrile contents.

On balance, acetonitrile exerted a deleterious effect on natively ordered porcine pepsin A, and promoted the establishment of intermolecular interactions. In what follows, it is proposed that increasing acetonitrile contents in the protein-solvent system above 5.13 – 5.70 M led to a progressive and pronounced decline in hydration and weakening of intramolecular hydrophobic interactions of porcine pepsin A, with subsequent protein unfolding.

In a globular protein, formation of hydrophobic core(s) is driven by removal of non-polar groups from water (or other polar solvents) (Spolar, *et al.*, 1989), in order to minimize the breakdown of water structure instigated by the presence of hydrophobic side chains (Finney, *et al.*, 1993; Head-Gordon, 1995).

Given that intramolecular hydrophobic interactions are favoured by a network of hydrogen-bonded water molecules located at the biomacromolecule surface, one must consider the effect of acetonitrile on the water structure when assessing its denaturing action on proteins. Water-acetonitrile mixtures have been subject of much controversy as regards their structural behaviour. However, it is well-established that the arrangement of hydrogen-bonded molecules in pure water is altered in aqueous solutions of non-electrolytes with a dual hydrophobic-hydrophilic character (Sirotkin, *et al.*, 2000). Moreover, results obtained by Catalán and collaborators (2003) for organic-aqueous solvent systems at mole fractions of acetonitrile lower than 0.7 (approximately 16.57 M) are in conflict with previous data claiming that addition of acetonitrile to water solely leads to occupation of cavities in the water structure, without establishing interactions with it. In fact, Robertson and Sugamori (1972) had already alluded to a disruption of water network in the close vicinity of acetonitrile molecules (Roberston & Sugamori, 1972 as quoted by Jamroz, *et al.*, 1993 and Kovacs & Laaksonen, 1991). Furthermore, results obtained by Bertie and Lan (1997) indicate that a slight weakening of the water structure results from acetonitrile addition. Venables and Schmuttenmaer (1998) reported on a loss in the tetrahedral water structure upon mixing with acetonitrile. And, more recently, Jerie and collaborators (2005) considered acetonitrile to be a structure breaker, albeit weak. Therefore, it is envisaged that, on raising acetonitrile contents (with a concomitant decrease in water contents) in the protein-solvent system, hydration networks at the protein surface were disturbed, resulting in distortion of the finely balanced interactions responsible for maintaining the native conformational state of porcine pepsin A. In other words, as a result of modifications in the water structure and hypothetical desorption of water molecules from the protein surface, hydrogen bonds as well as electrostatic and van der Waals interactions established between water and superficial groups were probably affected, provoking structural rearrangements. Most importantly, deterioration of the 'hydrophobic effect' – a dominant factor in the folding reaction of globular proteins (Chan & Dill, 1990; Pace *et al.*, 1996) – is believed to have followed from external water pressure relief and enhanced solubility of non-polar groups. The capacity for hydrophobic bonding of non-polar side chains, hitherto located in the protein interior, was weakened to such an extent that the porcine pepsin A globule was unravelled.

As indicated above, breakdown of secondary and tertiary structures in porcine pepsin A does not occur in unison (curves do not superimpose). This means that, for example, an acetonitrile concentration around 8.55 M was able to induce about 53.02% of the total change reported for native tertiary structure, but changed much less (approximately 28.89%) its secondary structure. Thus, intermediary forms are expected to have more damages in their tertiary packing than on their secondary structural elements. On the other hand, it is apparent that the final acetonitrile-induced unfolded state lacked, not only a rigid tertiary structural arrangement, but also regular and periodic structural elements. Disruption of the tertiary structure is surmised to have been directly provoked by the aforementioned drop in the capacity of non-polar side chains to establish hydrophobic interactions (with other non-polar side chains belonging to the same polypeptide chain), upon addition of organic solvent (Gekko, *et al.*, 1998). This is a reasonable assumption, taking into account the important contribution from self-association of non-polar groups as hydrophobic cores to the stabilization of the tertiary structure in natively ordered globular proteins (Vararattanavech, *et al.*, 2006). In regard to the secondary structure, its constituent units are known to be primarily determined by hydrogen

bonds involving the α-amide and α-carbonyl groups (Fernández, *et al.*, 2003). By virtue of being a weak competitor for intramolecular hydrogen bonds, it is hypothesized that acetonitrile did not seriously act on the secondary structure in a direct manner. Rather, the unpacking of hydrophobic side chains and ensuing expansion of conformations might have caused the drag of groups involved in the formation of regular structural elements, thereby causing decay of secondary structure. This presumption relies on proposals for a dynamical coupling between hydrophobic collapse and backbone burial during protein folding. In turn, backbone burial prompts engagement of amide-carbonyl hydrogen bonds in order to satisfy the hydrogen-bonding potential of buried main chain groups, giving rise to secondary structure (Fernández, *et al.*, 2003; Lesk, 2004; Lesser & Rose, 1990; Pace, *et al.*, 1996).

As previously mentioned, hydrophobic cores of porcine pepsin A were exposed on unfolding. In all likelihood, engagement of interactions between large hydrophobic surfaces of individual polypeptide chains accounts for the aggregative effects observed. Some degree of protein-protein association was reported even before the global conformational transition, which might hint at events of local disordering unobtainable by spectroscopic probes. Presuming that protein aggregation favoured exposure of hydrophilic groups to the surrounding medium in detriment of hydrophobic groups, protein insolubility at high concentrations of acetonitrile becomes understandable taking into consideration the following pieces of evidence. It is established that addition of acetonitrile to the medium causes diminished solubility of: (i) polar-charged side chains, especially in the presence of high contents of this organic solvent; (ii) polar-neutral side chains; and (iii) backbone peptide groups. Moreover, solubility of histidine, glutamic acid, aspartic acid and asparagine amino acids was recognized to be particularly low at high acetonitrile concentrations (Gekko, *et al.*, 1998). About 48.47% of the residues in the primary structure of porcine pepsin A are polar, and 17.2% are of the aforementioned types. In fact, a non-hydrogen bond donor character (Kang, *et al.*, 1994a; Kang, *et al.*, 1994b; Maroncelli, 1991; Olofsson, *et al.*, 2005) and a weak hydrogen bond acceptor capability (Venables & Schmuttenmaer, 1998) contribute to a weaker solvating power of acetonitrile in comparison to that of the water (or essentially aqueous media) (Ababneh, *et al.*, 2003). The absence of free water molecules may as well determine the weak solubility of polar groups in acetonitrile-rich media (Sirotkin, *et al.*, 2000). Indeed, despite the strong propensity for self-association of water molecules observed over a wide concentration range, Kovacs and Laaksonen (1991) confirmed the occurrence of complexes composed of one water molecule and two acetonitrile molecules linked by two weak hydrogen bonds in low-water media.

B. *Hydrodynamic Behaviour*

Assessment of modifications in the Stokes radius of a protein is helpful in understanding its denaturation and renaturation processes (*vide*, for instance, references: Parisi, *et al.*, 2003; Pattanaik, *et al.*, 1998). Accordingly, size exclusion chromatographic analyses of porcine pepsin A previously submitted to different solvent conditions were performed. The employment of such chromatographic technique was galvanized by its well-known sensitivity to small changes in the hydrodynamic properties of compact

proteins (Fish, *et al.*, 1970; Leach & Fish, 1977; Martenson, 1978).

Despite the well-proved applicability of size exclusion chromatography, not all gel matrices behave in a completely inert fashion, insomuch that proteins may adsorb to the gel phase under certain experimental conditions (Martenson, 1978; Scopes, 1994). Bearing in mind that partial adsorption delays elution and a protein will emerge as if it has a smaller molecular size than it actually has (Scopes, 1994), more or less specific binding between proteins and matrix may actually become a major obstacle for an accurate examination of the hydrodynamic behaviour of a protein (Martenson, 1978). In the research conducive to this dissertation, size exclusion chromatographic assays were carried out in *Superdex* medium, which is one of the most suitable types of media for working under harsh experimental conditions. Nonetheless, porcine pepsin A was noted to interact with the stationary phase under highly acidic conditions, for it was eluted with 10 mM sodium acetate at pH 3 to only a small extent [see Figure 4.14(a)]. A similar problem has already been reported by Campos and Sancho (2003) though the size exclusion chromatographic medium used by these authors was *Superose*. In order to try and surpass such shortcoming, the ionic strength of the mobile phase was increased by either raising the concentration of sodium acetate or changing the nature of the buffering compounds. Inclusively, sodium chloride was added to the original eluent (10 mM sodium acetate at pH 3) at final concentrations up to 1.00 M. Yet, the protein could not be successfully eluted from the column. Hence, it was concluded that protein-matrix binding was not of ionic nature. Further attempts to elute the protein from the chromatographic column involved replacing 10 mM sodium acetate at pH 3 by ultra-pure water as the mobile phase. By so doing, elution of porcine pepsin A was effectively accomplished. As it shall be seen later, elution of the protein was as well improved when acetonitrile was added to the mobile phase even at non-denaturing concentrations (or acetonitrile concentrations known not to induce noticeable conformational perturbations). In other words, a raise in environmental pH (until a specific limit) or hydrophobicity permitted elution of porcine pepsin A. This is symptomatic of hydrophobic interactions accounting for the adsorption of the protein to the matrix; although it is unlikely that hydrophobic cores are exposed to the stationary phase, when the protein assumes its natively folded conformational state.

As a general rule, it has been deemed that hydrophobic areas exposed to solvent are minimized upon protein folding (Spolar, *et al.*, 1989). However, recent investigations are suggestive of protein surfaces organized as patchworks of hydrophobic and hydrophilic regions. This should hold true for porcine pepsin A. From the molecular surface depiction in the Figure 4.13, it appears that the total hydrophobic surface area in the protein is considerable. This is supported by investigations on the interaction between porcine pepsin A and the synthetic drug 4', 6-diamidino-2-phenylindole (DAPI). It is worth adding that DAPI is positively charged at pH values lower than 12 and contains a hydrophobic moiety. Under conditions which should render electrostatic interactions negligible, experimental evidence pointed to an important role of hydrophobic interactions in the formation of porcine pepsin A - DAPI complexes at acidic pH (Mazzini, *et al.*, 1997). Furthermore, it was reported that native human pepsin 3b can only be eluted from reversed phase chromatographic columns with a concentration of organic modifier higher than that required to elute amyloglucosidase (97,000 Da), which was found to be the most hydrophobic protein among 33 different proteins analyzed in similar columns (Burton, *et al.*, 1988 as quoted by Jones, *et al.*, 1993). On account of

this observation and of 85.3% of structural identity between human pepsin 3b and porcine pepsin A, the belief that the latter enzyme in its native state is significantly hydrophobic gains consistency.

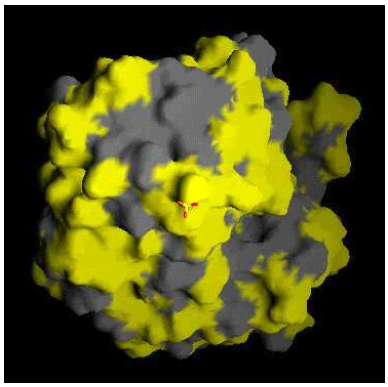

Figure 4.13 – Molecular surface of porcine pepsin A (PDB code *4pep*) coloured by hydrophobicity based on residue type. According to the default colouring scheme adopted, polar areas are in yellow and non-polar areas are in grey. This figure was generated by *GRASS* (*Graphical Representation and Analysis of Structure Server* available at http://trantor.bioc.columbia.edu/cgi-bin/GRASS/surfserv_enter.cgi) (Nayal, *et al.*, 1999).

Once elution of porcine pepsin A became practically unfeasible under native-like conditions, its elution position was predicted by interpolation of a calibration curve built for the chromatographic column. The relationship between the gel-phase distribution coefficient (K_{av}) of a set of calibrants (globular proteins in aqueous medium) and the base-10 logarithm of their respective molecular masses is shown in the Figure 4.14(b). The elution position for native porcine pepsin A was estimated to be around 26.37 mL.

Chromatograms depicted in the Figure 4.14(a) were obtained after typical periods of protein incubation in growing concentrations of organic solvent.

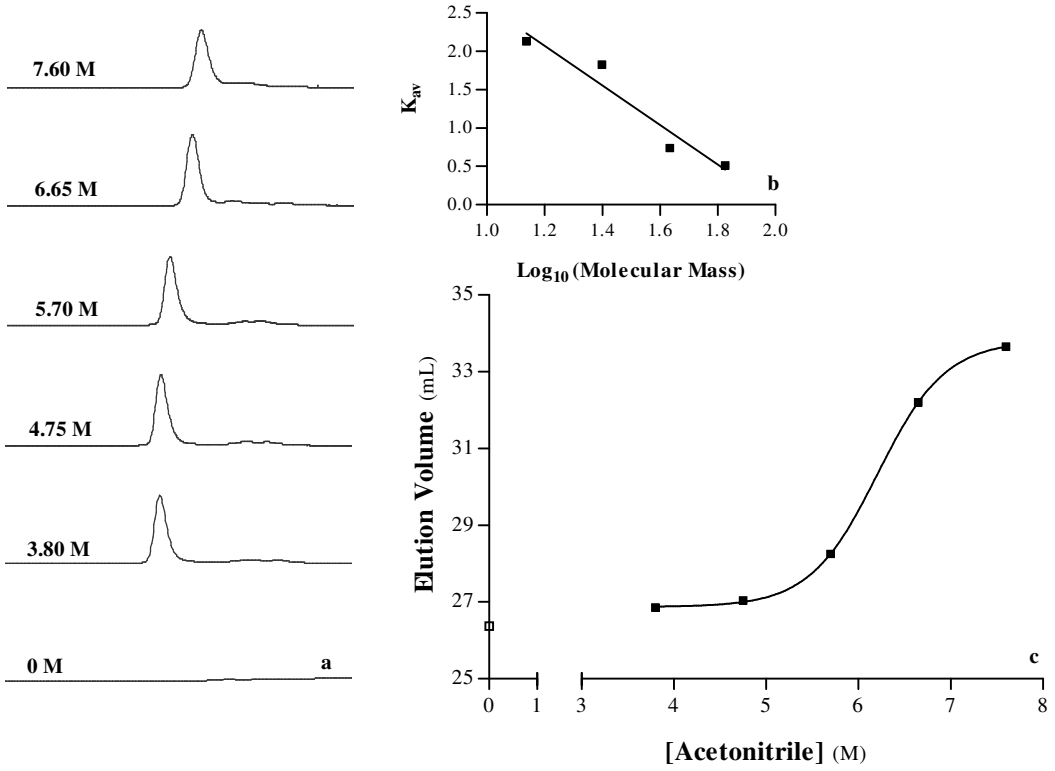

Figure 4.14 – **(a)** Size exclusion chromatographic profiles for porcine pepsin A in a *Superdex 75 HR 10/30* column after 1 h - incubation in growing concentrations of acetonitrile at 25ºC. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. **(b)** Calibration curve built from size exclusion chromatographic analyses of albumin from bovine serum (67,000 Da), ovalbumin from hen egg (43,000 Da), chymotrypsinogen A from bovine pancreas (25,000 Da), and ribonuclease A from bovine pancreas (13,700 Da) in the *Superdex 75 HR 10/30* column. (Data ceded by Anabela Pereira, MSc.) **(c)** Influence of acetonitrile concentration on the elution volume of porcine pepsin A (filled squares). Non-linear regression analysis yielded an R^2 equal to 0.99 (___). Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot. The empty square corresponds to the theoretical elution volume of porcine pepsin A in aqueous medium calculated from the calibration curve.

Several details on the elution profiles obtained in the range from 0.95 to 2.85 M acetonitrile were indicative of considerable protein adsorption to the matrix. At 0.95 M acetonitrile, the elution volume of porcine pepsin A was unrealistically high (in comparison to the elution volume estimated for the protein in aqueous medium), and the amount of protein still retained in the column after each chromatographic run was significant. Reductions both in the elution volume and in the amount of porcine pepsin A adsorbed on the stationary phase were observed up to 3.80 M of organic solvent. It should also be noted that peaks corresponding to elution of porcine pepsin A were broad and clearly asymmetrical in the range of acetonitrile concentrations from 0.95 to 2.85 M. It follows that differences in protein elution behaviours in this concentration range could not be attributed to a specific denaturing action of the organic solvent.

Retention of porcine pepsin A in the chromatographic column was assumed to be governed solely by differences in dimensions of hydrodynamic particles and pores at acetonitrile concentrations equal to and greater than 3.80 M acetonitrile. This assumption was made on account of maintenance of peak areas, an

extremely low amount of porcine pepsin A eluted upon the washing procedure and approximately symmetrical peaks at such concentrations of organic solvent, together with practically identical elution positions in the transition from 3.80 to 4.75 M of organic solvent [see Figure 4.14(a)].

In the Figure 4.14(c), the elution volume of porcine pepsin A was plotted as a function of acetonitrile concentration. In light of the above discussion, characterization of the hydrodynamic behaviour in the range from 0.95 to 2.85 M acetonitrile was inevitably precluded. Nonetheless, there was only a small, positive difference (approximately 0.48 mL) between the elution volume of porcine pepsin A in the presence of 3.80 M acetonitrile and the value predicted for the protein in aqueous medium. Striking changes in the elution position occurred only at concentrations greater than 4.75 M of organic solvent, which suggested a pronounced decline in the protein Stokes radius. It should be worth recalling that samples of porcine pepsin A in 7.60 M acetonitrile were centrifuged in order to isolate aggregates. This should account for the slight decrease in the peak area at this concentration of organic solvent. Size exclusion chromatographic analyses of porcine pepsin A in binary solvent systems containing acetonitrile at concentrations higher than 7.60 M were not performed due to limitations in the chemical stability of the support medium.

B.1. *Interpretative Remarks*

Taking into account that the hydrodynamic radius and volume of a protein increase upon unfolding (Lizarraga, *et al.*, 1978; Uversky & Ptitsyn, 1996), chromatographic data appear to be in disagreement with modifications in secondary and tertiary structural properties described in the previous section. Spectroscopic measurements suggested that porcine pepsin A underwent a global disordering transition above 5.13 – 5.70 M of organic solvent.

Herein, it is proposed that the drastic decrease in Stokes radius observed in the transition from 5.70 to 7.60 M acetonitrile was due to substantial dehydration and subsequent weak solvation by the organic solvent of, in particular, polar groups. The preceding suggestion follows from considering hydration layers as part of protein molecules and the need to account for them when analyzing hydrodynamic data (Koenig, *et al.*, 1975). Acetonitrile-promoted rearrangements in the spatial hydrogen-bonded network of water could impede the protein to hold its hydration shell in the conveniently tight manner required for sustaining the natively folded conformational state. Inclusively, water molecules attached to the protein surface might be stripped into the solvent (Gorman & Dordick, 1992; Khmelnitsky, *et al.*, 1991a; Simon, *et al.*, 1998). The ability of acetonitrile to remove the hydration water from enzyme surface was confirmed, for instance, by molecular dynamics simulations of surfactant-solubilized subtilisin BPN' (Yang, *et al.*, 2004). Furthermore, it is worth recalling that acetonitrile is only a hydrogen bond acceptor, and a very weak one (Ababneh, *et al.*, 2003; Kang, *et al.*, 1994a; Kang, *et al.*, 1994b; Maroncelli, 1991; Olofsson, *et al.*, 2005; Venables & Schmuttenmaer, 1998). In this light, acetonitrile molecules might not be able to fully supersede water molecules dislodged from the hydration shell, and there should be a decrease in the strength of solvent-protein interactions, together with a reduction in the residence time of solvent molecules at the protein surface. As such, it is reasonable to presume that addition of acetonitrile at concentrations equal to and

greater than 5.70 M induced striking damages in the original hydration structures covering the surface of porcine pepsin A formed in the step of dissolving it in 10 mM sodium acetate at pH 3, which favours the hypothesis previously raised (see section 1.A. '*Structural Properties*').

An integrated view of modifications in structural properties and hydrodynamic behaviour allows one to confidently propose that acetonitrile-induced global conformational disordering of porcine pepsin A was effectively triggered by a decrease in hydration. Besides, the hydrodynamic behaviour of the protein submitted to concentrations of organic solvent from 5.70 to 7.60 M seems to have been primarily determined by this factor rather than molecular expansion.

C. *Thermal Denaturation and Stability*

This section inquires on the influence of acetonitrile at apparent non-denaturing concentrations on the thermal denaturation process and stability of porcine pepsin A. Efforts in clarifying this issue entailed the employment of differential scanning calorimetry, in conjunction with circular dichroism spectroscopy.

Before considering thermal denaturation and properties of porcine pepsin A in binary aqueous mixtures of acetonitrile, one shall launch into a brief description of the data acquired in this scope for the target protein under standard conditions.

The heat absorption curve generated upon differential scanning calorimetric analysis of porcine pepsin A in 10 mM sodium acetate at pH 3, after 1 h - incubation at 25ºC, showed a sharp, single endothermic peak, which might convey a cooperative disordering transition (see Figure 4.15). This surmise is further validated by an average ratio of calorimetric enthalpy to van't Hoff enthalpy equal to 0.982. A cooperativity index close to 1 is expected for a two-state process (Haynie, 1998). Maximum excess heat capacity of porcine pepsin A in 10 mM sodium acetate at pH 3 was attained on average at 72.08ºC.

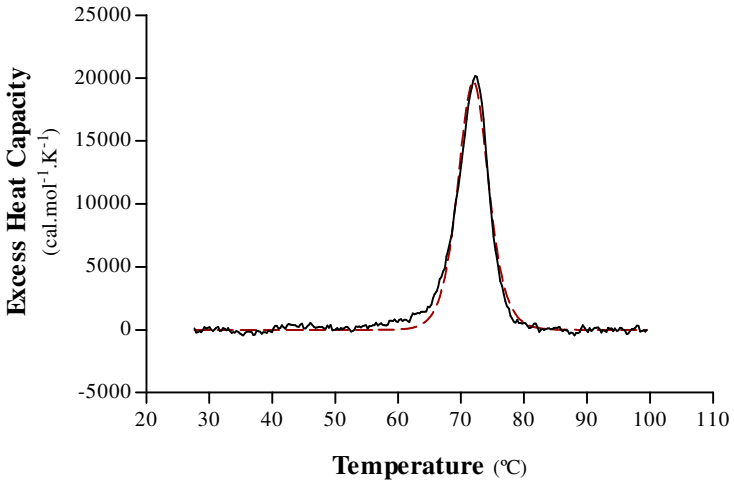

Figure 4.15 – Differential scanning calorimetric thermogram of porcine pepsin A in 10 mM sodium acetate at pH 3 subsequent to 1 h - incubation at 25ºC. Solid, black curve (___) corresponds to the experimental data obtained after subtraction of reference baseline, concentration normalization and subtraction of progress baseline. Dashed, red curve (_ _) represents the result of fit of the non-two-state model.

Absence of thermal effect in a second heating of the protein sample (after the first transition was completed and the sample was cooled back) is indicative of irreversibility of the global thermal denaturation process of porcine pepsin A in this experimental setting. A completely irreversible behaviour of thermal denaturation in acidic media has already been reported for pepsin (Tello-Solís & Hernandez-Arana, 1995). The nature of irreversible effects is not perceptible from the thermogram shape. However, formation of small aggregates and autolysis are two plausible sources of alterations in the folding properties of the polypeptide chain. Intermolecular association was visibly detected when porcine pepsin A in 10 mM sodium acetate pH 3 was incubated at a protein concentration 7.5-fold greater than the concentration of samples analyzed by differential scanning calorimetry and heated to 100ºC. A raise in temperature to values compatible with active conformations of porcine pepsin A is well-known to favour autolysis. Chemical deteriorative changes, including hydrolysis of peptide bonds at the carboxyl terminus of aspartic residues (Ahern & Klibanov, 1988; Volkin & Klibanov, 1991) or spontaneous deamidation of asparagine and glutamine residues (Tomazic & Klibanov, 1988; Volkin & Klibanov, 1991), could also have happened on exposure of unfolded conformations to high temperature values, affecting the reversibility of the conformational transition. Either way, proper folding of pepsin A, in particular, of its N-terminal domain is deemed to require the prosegment of the pepsinogen (Koelsch, *et al.*, 1994; Lin, *et al.*, 1992a; Privalov, *et al.*, 1981; Tello-Solís & Romero-García, 2001), so that complete recovery of the natively ordered and active state would be unattainable. Irreversibility precluded a direct analysis of differential scanning calorimetric data within the framework of classic equilibrium thermodynamics.

Thermal denaturation of native porcine pepsin A was further explored resorting to circular dichroism measurements in the amide region. Unlike differential scanning calorimetry, such approach

provides direct evidence for the structural modifications undergone by porcine pepsin A when submitted to a raise in temperature. As such, subsequent to a 1 h - incubation in 10 mM sodium acetate at pH 3 and 25ºC, and a 1:2.5 dilution with the same solvent, a spectrum of porcine pepsin A was collected at every 2ºC step in an increasing temperature gradient. The set of spectra generated are presented in the Figure 4.16(a). In order to gain a better understanding of modifications in the contents of secondary structural elements of porcine pepsin A, mean residue ellipticity at 208 and 216 nm was plotted against temperature [see Figure 4.16(b)].

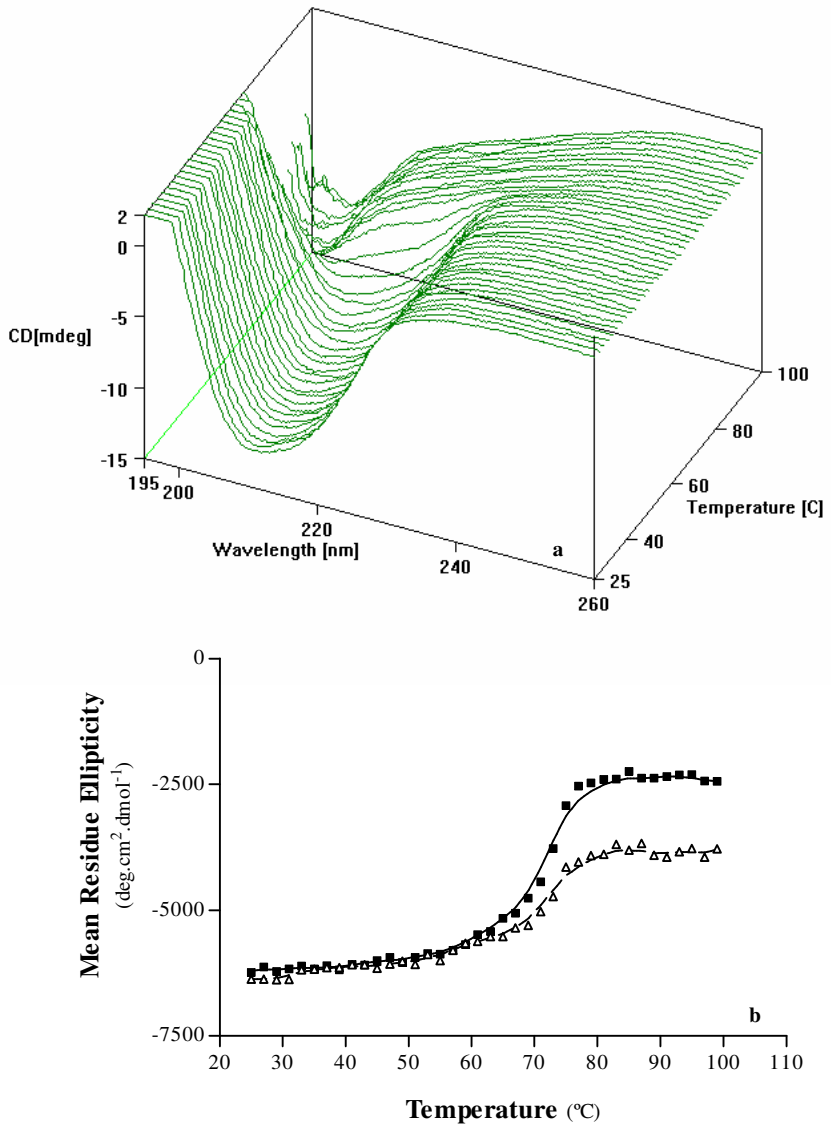

Figure 4.16 – **(a)** Far-UV circular dichroic spectra of porcine pepsin A in 10 mM sodium acetate at pH 3 in the temperature range from 25 to 99ºC. Spectra were acquired at every 2ºC step of the temperature gradient, after 1 h - incubation at 25ºC and a 1:2.5 dilution of the protein sample with the aqueous solvent. **(b)** Temperature-dependence of mean residue ellipticity of porcine pepsin A at 208 nm (empty triangles; _ _) and 216 nm (filled squares; ___).

It is remarkable to observe that the secondary structure of porcine pepsin A remained essentially intact until around 59ºC. On raising the temperature, absolute values of circular dichroic signal underwent a general decrease in the wavelength range from 195 to 230 nm. Nevertheless, it is worth highlighting the occurrence of an isodichroic point at about 205.2 nm. Reduction in the contents of β-structures and in the degree of helicity persisted up to around 79ºC. However, the final conformational state of heat-induced denaturation exhibited residual secondary structure (native or non-native), for there was still significant circular dichroic absorbance over the range from 205 to 230 nm. For example, mean residue ellipticity at 208 and 216 nm preserved 59.37% and 39.15% of the value typical for natively ordered porcine pepsin A, respectively.

A glance at Figure 4.16(b) might hint at a single-step decay of secondary structural elements, due to the sigmoidal relationship between mean residue ellipticity and temperature. This view is substantiated by the identification of an isodichroic point. And, it is in agreement with the previous proposal based on differential scanning calorimetric data.

The thermal structured-to-unstructured transition of porcine pepsin A as measured by circular dichroic absorbance at 208 and 216 nm afforded midpoint temperature values of 69.58 ± 0.692ºC and 70.63 ± 0.357ºC, respectively. These values are in fair accord with data from differential scanning calorimetric assays. In view of this, it is clear that both techniques monitored the same process.

As concerns the effect of acetonitrile, differential scanning calorimetric analyses of porcine pepsin A were performed upon incubation of the protein in binary aqueous mixtures containing organic solvent in the concentration range from 0.95 to 5.70 M. The incubation phase took place in the experimental setting previously described for analogous experiments in the absence of acetonitrile. A single endothermic peak was observed for every thermogram. Nonetheless, in differential scanning calorimetric traces for porcine pepsin A in the presence of acetonitrile concentrations equal to and higher than 1.90 M, the endotherm was followed almost immediately by exothermic heat effects due to aggregation of denatured conformations, which tended to occur at lower temperature values. A noisy post-transitional baseline was assigned to irregular convection effects owing to protein aggregation and precipitation within the calorimeter cell (Cooper, 1999). Aggregation and precipitation contributed to irreversibility of the heat-promoted disordering reaction. In the transition from 1.90 to 5.70 M acetonitrile, distance between exothermic and endothermic peaks became shorter. Distortion of endotherms due to irreversible phenomena hampered evaluation of the degree of cooperativity of the denaturation process, once determination of both calorimetric and van't Hoff enthalpy values would be unreliable.

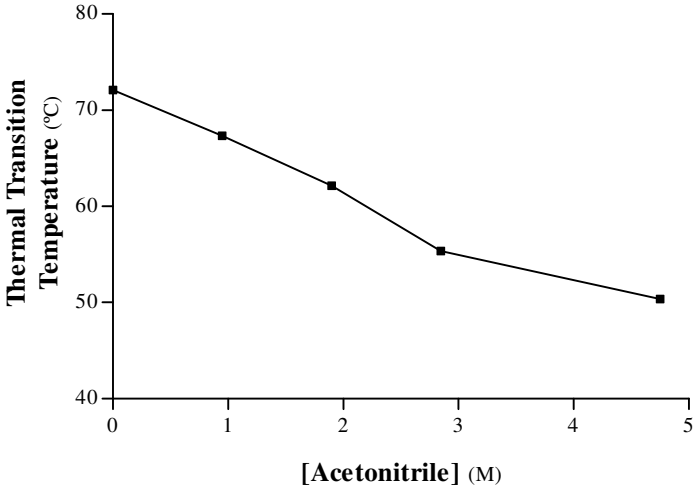

Figure 4.17 – Variations in thermal transition temperature of porcine pepsin A provoked by acetonitrile. Data were acquired from differential scanning calorimetric analyses of the protein after exposure to increasing concentrations of organic solvent during 1 h at 25ºC. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot.

As demonstrated in Figure 4.17, thermal transition temperature diminished as the concentration of acetonitrile was increased. Aggravated distortion of the endothermic peak yielded by differential scanning calorimetric analysis in the presence of 5.70 M acetonitrile impeded an accurate fit of the model and, thus, the transition midpoint could not be estimated. Thereafter, no more organic solvent concentrations were tested.

Circular dichroism spectroscopy in the far-UV region was employed to probe heat-provoked changes in the secondary structure of porcine pepsin A in the presence of selected concentrations of organic solvent, 1.90 and 5.70 M, which corresponded to concentration values, respectively, within the pre-transition region and at the lowest end of the transition region identified in the curve of the Figure 4.3(a). With this purpose, porcine pepsin A was submitted to an incubation period of 1 h in mixtures of 10 mM sodium acetate at pH 3 and acetonitrile at 25ºC. Before colleting spectra according to the procedural details provided elsewhere, samples were diluted to 1:2.5 with corresponding mixed solvent.

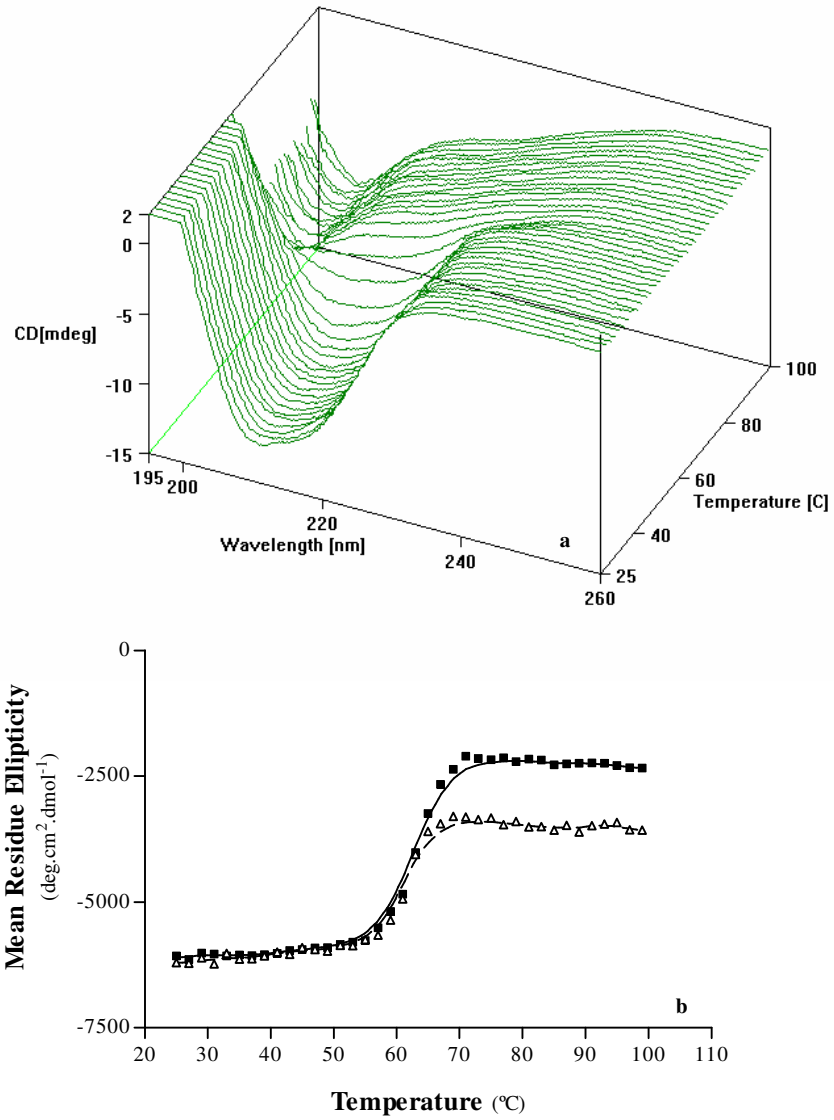

Figure 4.18 – **(a)** Far-UV circular dichroic spectra of porcine pepsin A in 1.90 M acetonitrile in the temperature range from 25 to 99ºC. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. Spectra were acquired at every 2ºC step of the temperature gradient, after 1 h - incubation in the binary aqueous mixture of acetonitrile at 25ºC and a 1:2.5 dilution of the protein sample with the mixed solvent. **(b)** Temperature-dependence of mean residue ellipticity of porcine pepsin A at 208 nm (empty triangles; _ _) and 216 nm (filled squares; ___).

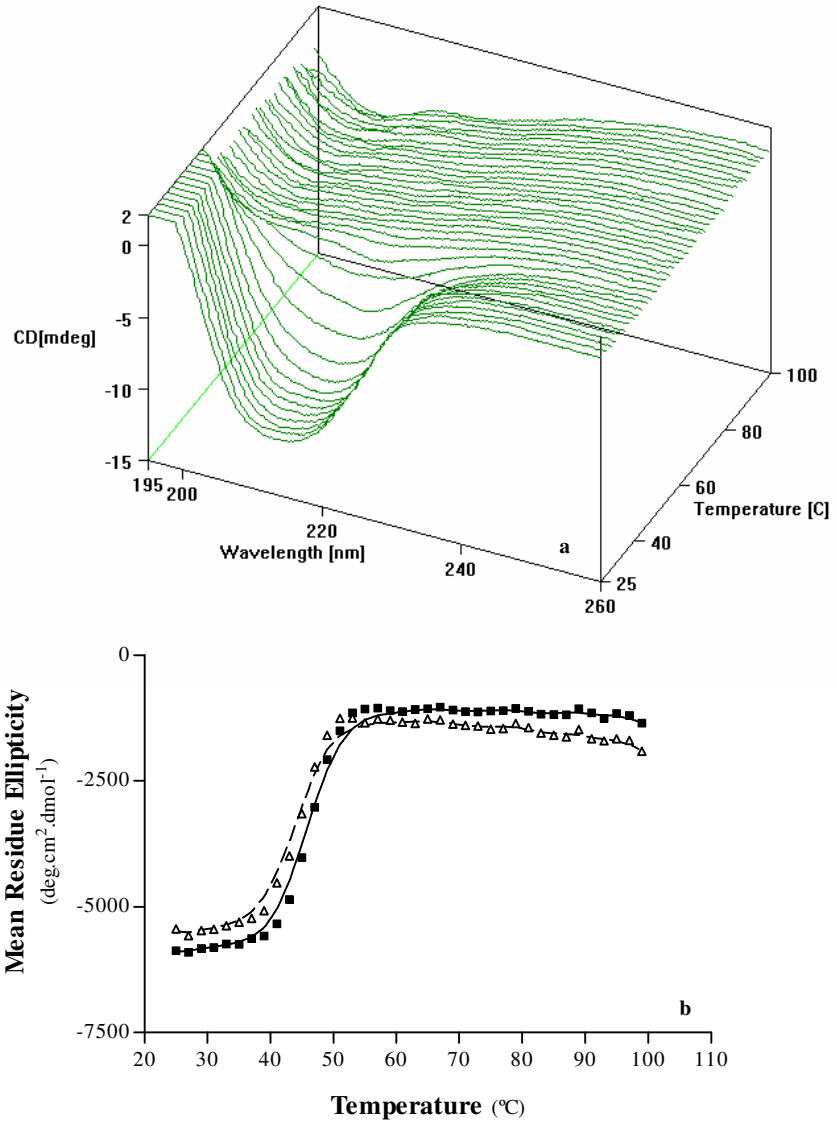

Figure 4.19 – **(a)** Far-UV circular dichroic spectra of porcine pepsin A in 5.70 M acetonitrile in the temperature range from 25 to 99ºC. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. Spectra were acquired at every 2ºC step of the temperature gradient, after 1 h - incubation in the binary aqueous mixture of acetonitrile at 25ºC and a 1:2.5 dilution of the protein sample with the mixed solvent. **(b)** Temperature-dependence of mean residue ellipticity of porcine pepsin A at 208 nm (empty triangles; _ _) and 216 nm (filled squares; ___).

As depicted in the Figure 4.18, a raise in temperature values did not promote noticeable alterations in the protein secondary structure of porcine pepsin A in the presence of 1.90 M acetonitrile until around 57ºC. Higher temperature values provoked a reduction in absolute values of circular dichroic signal for all wavelengths between 195 and 230 nm, with an isodichroic point at around 204.8 nm. In respect of thermal denaturation of porcine pepsin A in a binary aqueous mixture containing 5.70 M acetonitrile (see Figure 4.19), the conformational transition was onset at much lower temperature values *vis-à-vis* the thermal

disordering reaction in the absence and presence of 1.90 M acetonitrile. In the former case, a striking decrease in absolute values of circular dichroic signal started above 39ºC, and an isodichroic point was identified (as reported above for other solvent conditions) at about 202.4 nm. As seen in Figures 4.18(b) and 4.19(b), secondary structural transitions became more abrupt on switching from aqueous medium to aqueous-organic solvent systems. With regard to final denatured states, different degrees of unstructureness were brought about by different contents of acetonitrile in the medium. When the solvent system contained 1.90 M acetonitrile, a denatured conformational state was attained at temperature values above 71ºC, whose spectral features were consistent with residual helicity and β-structure. Low- and high-temperature conformational ensembles in the absence and presence of 1.90 M acetonitrile were comparable; although porcine pepsin A sampled slightly more disordered conformations in the latter solvent condition at the higher bound of the temperature range spanned. On the other hand, porcine pepsin A in 5.70 M acetonitrile adopted a denatured state at approximately 53ºC. Of relevance is the fact that the corresponding spectra were closer to the typical spectrum of a random coil - like conformation in comparison with spectra of thermally denatured states in aqueous medium and in the presence of a lower concentration of acetonitrile.

For both mixed solvent systems, thermal transition curves obtained for porcine pepsin A at the two wavelengths within the amide region were synergetic. On the basis of relatively sharp and sigmoidal curves measured by far-UV circular dichroism and the identification of isodichroic points, it appears that heat-induced secondary structural transitions in the presence of 1.90 and 5.70 M acetonitrile were cooperative and conformed to a two-state model. However, absence of discrete intermediates could not be credibly verified by calorimetric data. Thermal transition temperature values, as indicated by the midpoint of denaturation curves determined by far-UV circular dichroic absorbance at 208 and 216 nm, were respectively: 61.04 ± 0.226ºC and 62.60 ± 0.145ºC in the presence of 1.90 M acetonitrile; and 44.06 ± 0.237ºC and 46.05 ± 0.095ºC in the presence of 5.70 M acetonitrile. These values are in satisfactory agreement with those estimated from differential scanning calorimetric thermograms.

C.1. *Interpretative Remarks*

To sum up, a decrease in the thermal transition temperature of porcine pepsin A was induced by acetonitrile in the concentration range from 0.95 to 5.70 M [see Figures 4.15, 4.16(b), 4.17, 4.18(b) and 4.19(b)]. This finding suggests a strong reduction in protein thermal stability on addition of organic solvent; though no severe conformational damages were detected through spectroscopic methods within this interval of acetonitrile concentrations [see Figures 4.3(a), 4.6(a) and 4.10]. Thus, it is proposed that acetonitrile impacted interactions that stabilize the native folding of the enzyme even before inducing global denaturation. In harmony with previous proposals, it is suggested that, during the incubation step prior to the differential scanning calorimetric analysis, acetonitrile in the concentration range from 0.95 to 5.70 M would mainly perturb water molecules at the protein surface; so that the balance of non-covalent interactions which assures the natively ordered state would be affected and protein conformations would become more susceptible to heating.

Although low-temperature conformational states in binary aqueous mixtures of acetonitrile were native-like, the presence of organic solvent did not only render the protein more thermosensitive, but it also impacted the structure of denatured states and reduced their solubility. Thermally induced conformations in aqueous conditions and in the presence of 1.90 M acetonitrile were comparable, but it was clear that they did not conform to the random coil model. Indeed, interactions between extensive fractions of protein residues have been reported to persist upon heat-induced disordering transitions in the absence of denaturants, insomuch as their end-points often display residual structure (Arnold, *et al.*, 1996; Evans, *et al.*, 1991; Kreimer, *et al.*, 1995; Lazaridis & Karplus, 1999; Seshadri, *et al.*, 1994; Sosnick & Trewhella, 1992). Conversely, in the presence of 5.70 M acetonitrile, high-temperature conformations further unfolded, *i. e.*, underwent a much greater loss of native-like secondary structure, given that their corresponding spectra were closer to a spectrum of a random coil - like state.

Even in the absence of denaturants, heat-induced denaturation proceeds via a complex mechanism. On heating a protein-solvent system, water structure breaks down (Royer, 2005), water molecules bound to a protein molecule are displaced, and the strength of non-covalent (intramolecular) interactions, including hydrogen bonds, electrostatic and van der Waals interactions, and of the hydrophobic effect, is affected to different extents (Ross & Rekharsky, 1996; Sterner & Liebl, 2001; Vieille & Zeikus, 2001; Volkin & Klibanov, 1991). At sufficiently high temperature values, extensive intramolecular motions take place (Ahern & Klibanov, 1988), leading to weakening and breakage of hydrogen bonds (Ross & Rekharsky, 1996; Scheraga, *et al.*, 1962), along with disruption of van der Waals interactions between hydrophobic residues (Privalov & Gill, 1988 as quoted by Maki, *et al.*, 2005) – two major sources of the thermal effect, which results in protein expansion (Royer, 2005). As a protein unfolds, hydrophobic areas become more easily accessible to the surrounding medium. In the presence of a second denaturing factor such as acetonitrile, the contact between non-polar groups in the protein and the organic solvent could contribute to more abrupt thermal denaturation transitions and to a higher degree of conformational disorder in their end-points.

In conclusion, steeper heat-promoted order-to-disorder transitions and a reduction in the thermal transition temperature of native porcine pepsin A upon incubation in binary aqueous mixtures of acetonitrile, together with an aggravation in the degree of unstructureness of the thermally denatured state in the presence of 5.70 M of organic solvent, give evidence of cumulative effects by acetonitrile and temperature.

D. *Peptidolytic Activity*

In this section, the effect of growing concentrations of acetonitrile on the hydrolysis of the synthetic peptide, H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe, catalyzed by porcine pepsin A will be considered. Appraisal of changes in the pepsinolytic activity may aid in surveying to which extent the active site region and its neighbourhood retain their structural integrity upon exposure to specific denaturing conditions. In other words, enzymatic activity may be regarded as an indicator of stability of the active site and other structural elements implicated in catalysis, as long as modifications in the hydrolytic rate can be

exclusively ascribable to conformational adjustments.

The dependence of the rate of porcine pepsin A - catalyzed hydrolysis of the synthetic peptide on the concentration of acetonitrile was gauged after 1 h - incubation of the enzyme at 25ºC in mixed solvent systems with corresponding volume ratios of aqueous (10 mM sodium acetate at pH 3) and non-aqueous components.

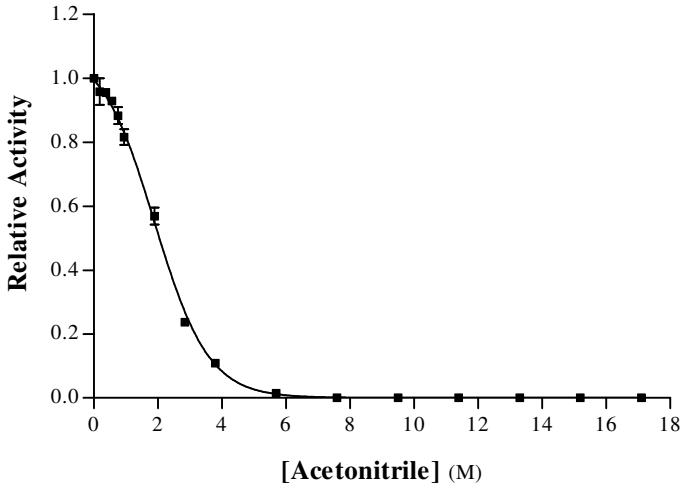

Figure 4.20 – Effect of increasing concentrations of acetonitrile on the rate of hydrolysis of the synthetic peptide, H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe, catalyzed by porcine pepsin A at 25ºC. Peptidolytic assays were performed after 1 h - incubation of the enzyme at 25ºC in a mixed solvent system with corresponding acetonitrile concentrations. Aqueous components of both incubation and reaction media were titrated to pH 3. Non-linear regression analysis yielded an R^2 equal to 0.99. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot.

As seen in the Figure 4.20, the rate of hydrolysis of the peptide bond, *p* – nitro – Phe – Nle, catalyzed by porcine pepsin A in binary aqueous-organic mixtures decreased with an increase in acetonitrile contents. 50% of maximum activity (activity shown in the absence of acetonitrile) was attained at 1.90 ± 0.071 M of organic solvent. Catalytic activity was extinguished above 5.70 M acetonitrile.

In addition, time-dependence of acetonitrile-provoked alterations in the peptidolytic activity by porcine pepsin A was evaluated. For this purpose, rates of porcine pepsin A - catalyzed hydrolysis of the synthetic peptide, measured in the presence of selected concentrations of organic solvent after 1 h - incubation of the enzyme in aqueous-organic mixtures with corresponding concentrations of acetonitrile, were compared with similar data acquired after exposure to parallel conditions for 0 and 24 h.

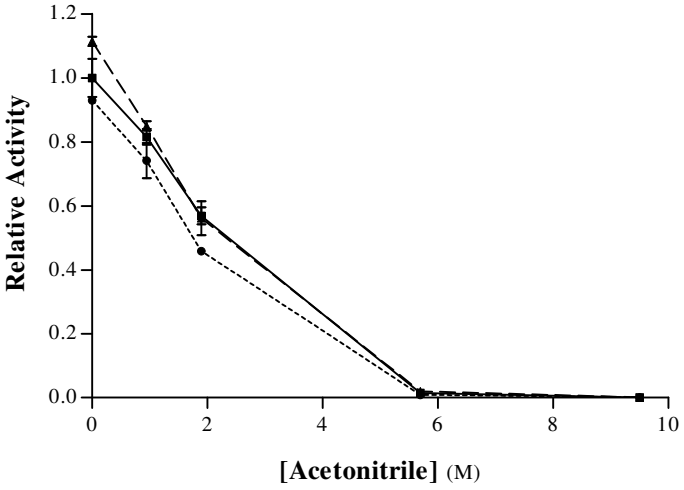

Figure 4.21 – Time-dependence of acetonitrile-induced changes on the rates of porcine pepsin A - catalyzed hydrolysis of the synthetic peptide, H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe. Peptidolytic assays were performed after 0 h - (triangles; _ _), 1 h - (squares; __) and 24 h - incubation (circles; ….) of the enzyme at 25ºC in a mixed solvent system with corresponding acetonitrile concentrations. Aqueous components of both incubation and reaction media were titrated to pH 3. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot.

Figure 4.21 shows an overall decrease in peptidolytic activity in the transition from a 0 h - to a 24 h - incubation in both the aqueous medium and binary aqueous-organic mixtures with 0.95 and 1.90 M acetonitrile. However, regardless of the time of incubation, porcine pepsin A - catalyzed hydrolysis of the synthetic peptide was negligible or absent in the presence of 5.70 and 9.50 M of organic solvent. It is noteworthy that porcine pepsin A - catalyzed hydrolytic reaction did not occur at the latter concentration of organic solvent even in the absence of an incubation step, although 9.50 M acetonitrile did not instantaneously induce a marked destruction of native secondary structural elements [see Figure 4.4(a, b)].

D.1. *Interpretative Remarks*

Peptidolytic activity of porcine pepsin A decayed with a raise in the contents of acetonitrile in both incubation and reaction media up to 5.70 M (see Figure 4.20). This is not surprising since organic solvents are often reported to diminish rates of enzyme-catalyzed reactions (Klibanov, 1997; Yang, *et al.*, 2004).

A decreasing trend of hydrolytic rates was concomitant with a deleterious effect of acetonitrile on the thermal stability of the enzyme [see Figures 4.15, 4.16(b), 4.17, 4.18(b) and 4.19(b)]. Nevertheless, intrinsic spectroscopic properties and hydrodynamic behaviour of porcine pepsin A did only undergo considerable modifications above 5.13 – 5.70 M of organic solvent [see Figures 4.3(a), 4.6(a), 4.10 and 4.14]. Taken together, these results reveal that, in the presence of growing concentrations of acetonitrile, peptidolytic activity started to decrease before the onset of global unfolding. A reasonable explanation for this finding could be given by the general belief that the active site region and structural elements with a

specific participation in catalysis, display particularly high sensitivity to environmental perturbations in comparison to the rest of the enzyme structure (Tsou, 1998a). And, in consonance with the proposal previously put forth in the context of the impact of acetonitrile on thermal stability (see section 1.C. '*Thermal Denaturation and Stability*'), this organic solvent at concentrations lower than 5.70 M may affect non-covalent interactions which sustain the natively folded and active conformational state, giving rise to localized unfolding events prior to global denaturation.

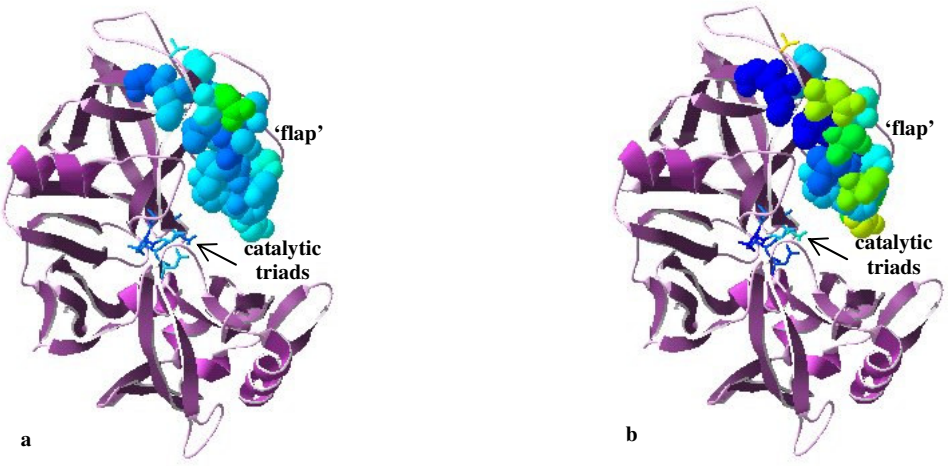

Figure 4.22 – Flexibility and accessibility of the 'flap' and catalytic triads of porcine pepsin A (PDB code *4pep*). The main chain trace is shown in cartoon format. Residues from the 'flap' and catalytic triads are coloured according to **(a)** *B*-factor and **(b)** accessibility. The colour scheme includes spectral colours: on one end, dark blue stands for low relative surface accessibility and low *B*-factor; on the opposite end, red stands for relative surface accessibility equal to or greater than 75% and high *B*-factor. These figures were generated and rendered by means of the interface *Swiss-PdbViewer 3.7*.

A strong correlation has been drawn between conformational fragility and flexibility (Tsou, 1998a; Tsou, 1998b). And, in fact, the catalytic mechanism of pepsin A is dependent on the residues and dynamics of a segment in the proximity of the active site, the 'flap'. This loop is formed by two antiparallel β-strands, comprises residues in the positions from 70 to 85 and includes four glycine residues, which are known to contribute to structural flexibility. In contrast to the catalytic triads and binding subsites, high *B*-factor values are observed in some residues of the 'flap', confirming its mobility in the native conformational state [see Figure 4.22(a)]. This structural element projects over the active site and participates in substrate binding. The 'flap' moves as a whole and, in so doing, alters its own chain conformation, particularly, in the region of the turn Gly76 – Gly78 (Andreeva, *et al.*, 1995; Okoniewska, *et al.*, 1999). In line with this, one could expect enhanced velocity of the enzyme-catalyzed reaction on addition of acetonitrile in response to, for instance, improved flexibility of the 'flap' caused by local disordering phenomena. Indeed, a few residues in the 'flap' exhibit a relatively significant solvent accessible surface area in contrast to the catalytic triads in the native conformational state [see Figure 4.22(b)]. Nonetheless, it is important to emphasize that optimal catalytic activity of an enzyme such as pepsin A, whose biological role requires both high catalytic efficiency and broad specificity (Dunn, *et al.*, 1986; Welinder, 1988), entails a convenient balance between conformational flexibility and rigidity (Szeltner & Polgár, 1996). A specific level of stiffness is generally needed to assure an

adequate geometry for substrate binding to enzyme and, hence, to allow efficient catalysis (Fields, 2001; Gladilin & Levashov, 1998; Szeltner & Polgár, 1996). In respect of aspartic proteinases, it has been noted that a proper and productive binding of substrate demands a fixed position and definite conformation of the 'flap' (Andreeva, *et al.*, 1995), together with a rigid active site [see Figure 4.22(a)] owing to entropic reasons (Davies, 1990; Lin, *et al.*, 1992b; Prasad & Suguna, 2002; Szeltner & Polgár, 1996). Denaturant-induced local changes at the 'flap' might have exposed the active site and rendered it more susceptible to the influence of the surrounding solvent. Moreover, given that highly specific substrates are very sensitive to alterations in the active site (Gladilin & Levashov, 1998), it is likely that any modification in residues involved in catalysis should result in a reduction in catalytic efficiency, or even hamper catalysis (Fields, 2001). Major modifications in the position of subsites responsible for recognition of substrates could result from, for instance, small changes in the relative position of secondary structural elements (Cooper, 2002). In light of these considerations, activity measurements, which mostly depend on a limited region of the molecule, might actually consist of a tool sensitive to the effect of low amounts of denaturants.

To sum up, excessive flexibility of the 'flap' and active site region provoked by acetonitrile at concentrations lower than 5.70 M (after incubation of the protein in aqueous-organic mixtures) might have consisted of a source of perturbation, insomuch that the structure might have become looser and impeded the formation of a productive enzyme-substrate complex. Above this concentration of organic solvent, adequate conformations and relative orientations of the active site and 'flap' might have been affected to such an extent that porcine pepsin A - catalyzed hydrolysis of the synthetic hexapeptide did not occur (see Figure 4.20). The proposal for a defeat in stability of the active site region and 'flap' as a primary mechanism responsible for inactivation is withstood by a concomitant drop in proteolytic activity and thermal stability below 5.70 M acetonitrile upon incubation of the enzyme in aqueous-organic mixtures, and it is reinforced by the simultaneity of full inactivation and onset of global denaturation above this concentration of organic solvent.

It is valuable to add that removal of water from the microenvironment of catalytic apparatus could have *per se* a seminal role in inactivation. Changes in the solvation water may affect catalytic activity, not only by modifying the stabilization of the overall structure, but also by affecting specific details of the active site hydration. In aspartic proteinases, a number of conserved water molecules aid in fixing the active conformation of the 'flap' during substrate binding, and in sustaining the geometry of active site crucial for catalysis (Andreeva, *et al.*, 1995; Andreeva & Rumsh, 2001; Polgár, 1987; Prasad & Suguna, 2002; Topol, *et al.*, 1995; Veerapandian, *et al*., 1992). In the last analysis, porcine pepsin A is a hydrolase, whose catalytic mechanism is dependent on a water molecule acting as a nucleophilic group (Andreeva & Rumsh, 2001; Polgár, 1987; Polgár, 1999; Prasad & Suguna, 2002).

Yet, inhibition cannot be completely ruled out as a mechanism responsible for reduction in enzymatic activity. Actually, active sites have been surmised to be important binding sites of organic solvents (Dennis, *et al.*, 2002; Maurel, 1978; Ringe & Mattos, 1999), and acetonitrile is not an exception (Gupta, *et al.*, 2000). Yang and co-workers (2004) have found that penetration of acetonitrile molecules into surface crevices and, especially into the active site, together with replacement of weakly bound water molecules in this region, ensues from displacement of hydration water of surfactant-solubilized subtilisin BPN' by the

organic solvent. Therefore, it is reasonable to hypothesize that the influence of acetonitrile on enzymatic activity was due to disturbance of hydration and conformational destabilization in conjunction with competitive inhibition.

The well-established preference of pepsin A for cleaving substrates between two hydrophobic residues (Fruton, 1970; Fruton, 2002) and the identification of several hydrophobic pockets around the active site (Andreeva, *et al.*, 1984) lend support to a potential competitive inhibitory mechanism by molecules of organic solvent located at the active site region. Early on, Tang (1965) argued that hydrocarbon groups of a series of aliphatic alcohols could compete with the substrate for the binding site in the enzyme and, subsequently, act as competitive inhibitors. Additionally, inhibition was found to be more pronounced in the presence of alcohols with longer hydrocarbon groups (Tang, 1965). Meanwhile, strong inhibitory properties of ethanol against porcine pepsin A were ascertained (Antonov, 1977; Dalgalarrondo, *et al.*, 1995). In 1988, Welinder performed a study on pepsin-catalyzed proteolysis at pH 2 – 3 in the presence of 20% (v/v) methanol, ethanol, 2-propanol and acetonitrile at 22 and 37ºC, after a 18 h - incubation in the different solvent conditions. Aside from having observed a trend of decreasing hydrolytic rates with an increasing number of carbon atoms in the alcohol molecules in agreement with observations by Tang (1965); Welinder (1988) postulated that competitive inhibition is also in operation as far as acetonitrile-dependent changes in catalytic activity of pepsin A are concerned. However, inhibition was surmised not to have a role in inactivation of pepsin by 20% (v/v) (or 3.80 M) acetonitrile as prevailing as in the case of its inactivation by 20% (v/v) 2-propanol. The following pieces of evidence uphold this assumption: (i) disparity between the small size of the hydrocarbon group in an acetonitrile molecule and the dimension of the extended substrate binding site of pepsin A and aspartic proteinases in general (Andreeva, *et al.*, 1984; Antonov, 1977; Sampath-Kumar & Fruton, 1974); and (ii) greater resemblance of hydrophobic residues that determine substrate specificity of pepsin with compounds with three carbon atoms (*e. g.*, 2-propanol) than with compounds with two carbon atoms (*e. g.*, acetonitrile and ethanol) (Welinder, 1988). A weak, non-specific interaction with the active site is likely aided by the aprotic character of acetonitrile and its poor hydrogen bond acceptor capability.

2. *CHAOTROPE-INDUCED DENATURATION OF PORCINE PEPSIN A*

Compounds such as guanidine hydrochloride and urea are powerful inductors of protein denaturation, being commonly referred to as *chaotropic agents*. The potential of guanidine hydrochloride and urea for perturbing protein structure has been known for close to a century (Timasheff & Xie, 2003). Their employment has become commonplace in studies aiming at characterizing folding and unfolding reactions and evaluating conformational stability (Ahmad, *et al.*, 2005; Akhtar, *et al.*, 2002; Cerasoli, *et al.*, 2002; Datta, *et al.*, 2003; Del Vecchio, *et al.*, 2002; Mazzini, *et al.*, 2002; Pace, 1986; Shukla, *et al.*, 2005; Stepanenko, *et al.*, 2004).

Guanidine hydrochloride (CH_6N_3Cl) is a monovalent salt. It is ionized in aqueous solutions, splitting into the guanidinium cation ($CH_6N_3^+$), whose positive charge is delocalized over the planar structure, and the chloride anion (Cl^-) (Monera, *et al.*, 1994). The guanidinium ion is structurally homologous to the arginine side chain. By virtue of this, the charged denaturant might be a useful tool in protein folding studies (Mason, *et al.*, 2003). Urea (CH_4ON_2) is a neutral compound (Monera, *et al.*, 1994), which is very similar to the guanidinium ion from a structural viewpoint (Shukla, *et al.*, 2005). Both chaotropic agents are highly soluble in water (Pace, *et al.*, 2005; Vanzi, *et al.*, 1998), and capable of engaging more than one hydrogen bond (Dunbar, *et al.*, 1997). In respect of the guanidinium ion, hydrogen bonds are mainly formed in the molecular plane (Dempsey, *et al.*, 2005). In solution, urea exhibits higher hydrogen-bonding capacity than water (Pace, *et al.*, 2005; Soper, *et al.*, 2003). The non-electrolyte forms hydrogen bonds at both amine and carbonyl head groups (Soper, *et al.*, 2003). Both chaotropes exhibit weak first hydration shells (Washabaugh & Collins, 1986). Guanidinium ion and urea display a dual nature, being able to interact with polar and non-polar groups (Vanzi, *et al.*, 1998).

For long, two different perspectives have driven research on how chaotropic agents interfere with interactions that stabilize proteins causing unfolding. On one hand, an *indirect mechanism* of action has been considered, according to which addition of a denaturant to the protein solvent-system provokes modifications in the structure of the hydrogen-bonded network of water around hydrophobic groups in proteins, leading to an increase in their solubility and weakening the hydrophobic effect (Caballero-Herrera, *et al.*, 2005; Modig, *et al.*, 2003; Nozaki & Tanford, 1963; Vanzi, *et al.*, 1998). On the other hand, suggestions have been made that promotion of protein disordering by these reagents mainly stems from their preferential binding to groups freshly exposed by unfolding. This is the so-called *direct mechanism* (Courtenay, *et al.*, 2001; Möglich, *et al.*, 2005; Schellman, 2003; Timasheff & Xie, 2003; Vanzi, *et al.*, 1998). Accessibility of a larger number of binding sites in the unfolded as compared to the folded state explains stabilization of the former in detriment of the latter, *i. e.*, favours protein unfolding (Möglich, *et al.*, 2005; Pace, 1986; Shortle, 1996). Mounting evidence points to the possibility of both mechanisms being in operation during chemical denaturation (Bennion & Daggett, 2003; Del Vecchio, *et al*., 2002; Möglich, *et al.*, 2005; Vanzi, *et al.*, 1998; Zou, *et al.*, 1998).

Despite a plethora of experimental and simulation studies on the impact of guanidine hydrochloride and urea on protein conformation and stability, molecular and physical bases of the mechanisms whereby these two chaotropes act are still not completely clear (Bennion & Daggett, 2003; Dunbar, *et al.*, 1997; Kesavulu, *et al.*, 2005; Modig, *et al.*, 2003). Similarly, the extents to which different protein groups contribute to the biomacromolecule sensitivity against guanidine hydrochloride and urea do also remain to be precisely understood. Confrontation of wide-ranging information about the structure of porcine pepsin A and its behaviour during chaotrope-induced denaturation should be useful for raising some hypotheses about this issue.

In the ongoing subchapter, data on conformational changes induced in porcine pepsin A by guanidine hydrochloride and urea amassed during the research conducive to this dissertation will be

described. Tentative interpretations of the observed effects will be built on the basis of current knowledge about the mechanism of action of these denaturants. It is of relevance to recall that the target protein assumes its natively folded state in the reference environmental setup used in this work, several complementary approaches are being simultaneously applied and a wide range of conditions are being examined. This should allow a better understanding of the response of native porcine pepsin A to guanidine hydrochloride and urea than that provided by ancient studies, and might aid in withdrawing inferences about the conformational stability of the protein.

Except for the experimental work presented in the section 2.E. '*Induction of Denaturation under Non-Standard Experimental Conditions*', the protein under study was submitted to increasing concentrations of each chaotrope for 1 h and 25ºC before collection of the data. Unless otherwise stated, an aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures.

A. *Structural Properties*

A.1. *Secondary Structure*

The effects of guanidine hydrochloride and urea on the structural units supportive of pepsin secondary structure are illustrated in the Figure 4.23. Due to high circular dichroic absorbance by both chaotropes below 210 nm, spectra could only be analyzed in the wavelength range from 210 to 260 nm.

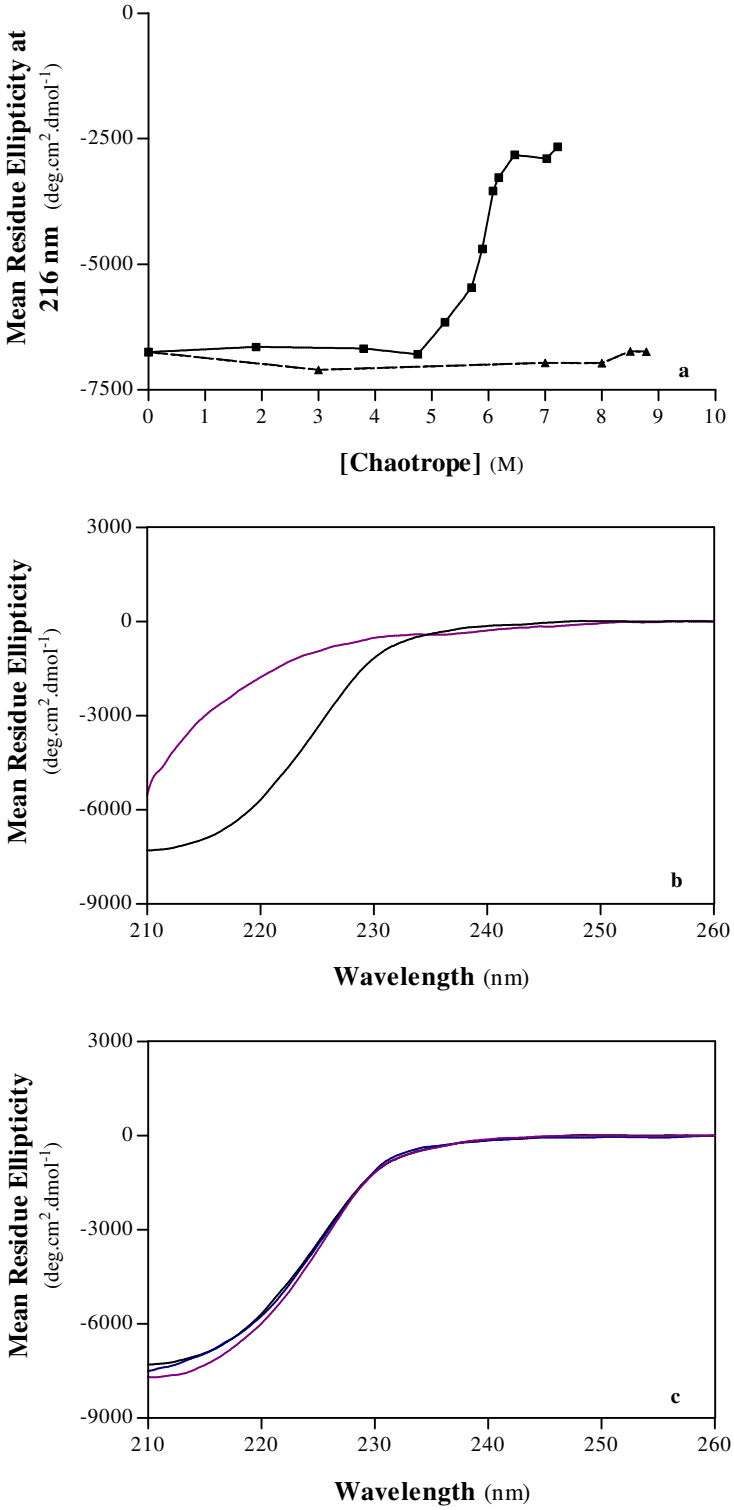

Figure 4.23 – **(a)** Dependence of the mean residue ellipticity of porcine pepsin A at 216 nm on guanidine hydrochloride (squares; ___) and urea (triangles; _ _) molarity. In respect of the curve corresponding to the impact of urea on the pepsin secondary structure, the line was merely used to connect the symbols. Data were obtained from far-UV circular dichroic spectra, which were recorded after 1 h of exposure of porcine pepsin A to varying concentrations of each chaotrope at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. **(b)** Far-UV circular dichroic spectra of porcine pepsin A collected after 1 h - incubation at 25ºC in aqueous medium (___) and in the presence of 7.22 M guanidine hydrochloride (___). **(c)** Far-UV circular dichroic spectra of porcine pepsin A collected after 1 h - incubation at 25ºC in the absence (___), and in the presence of 3.00 M (___) and 8.79 M (___) urea.

Figure 4.23(a) shows that no appreciable alterations on regular structural elements occurred, when guanidine hydrochloride was added to the solvent system at concentrations equal to 1.90, 3.80 or 4.75 M. Outside the 0 – 4.75 M range of chaotrope concentrations, large-scale changes in the secondary structure became noticeable. A raise in the amount of perturbant in the incubation medium up to about 6.46 M led to a general increase in values of circular dichroic signal at every wavelength between 210 and 230 nm. This observation is supportive of a drastic debilitation of the overall secondary structure. On fitting a sigmoidal model to the curve in the Figure 4.23(a) (with an R^2 equal to 0.99), the transition midpoint concentration was estimated to be 5.86 ± 0.027 M.

It is clear from the Figure 4.23(a) that the denatured macrostate isolatable at concentrations equal to and above 6.46 M guanidine hydrochloride retained a remarkable amount of ordered structure (native or non-native). This is suggested by the significant negative spectroscopic signal observed between 210 and 230 nm for porcine pepsin A in the presence of 7.22 M guanidine hydrochloride [see Figure 4.23(b)]. Under the selected experimental conditions, the conformational state sampled by the protein at the highest concentration of this chaotrope preserved 39.44% of the value of mean residue ellipticity at 216 nm typical for the natively ordered state.

With regard to urea, it proved to be unarguably inefficient in disordering porcine pepsin A in the environmental setup used in this study [see Figure 4.23(a)]. Indeed, superposition of spectral traces obtained in the amide region corresponding to 0 and 8.79 M urea indicated that, in principle, the protein conserved a comparable degree of secondary structural organization upon exposure to the highest denaturant concentration tested [see Figure 4.23(c)]. It was further detected that the magnitude of the negative band in the far-UV circular dichroic spectrum of porcine pepsin A underwent slight increases in the presence of 3.00, 7.00 and 8.00 M urea in relation to spectra of control samples in aqueous medium. From the urea concentrations explored here, the lowest value of mean residue ellipticity at 216 nm was observed at 3.00 M of chaotrope. Taking into account that circular dichroic absorbance in the deep-UV region mainly originates from the spatial arrangement of amide groups (Besley & Hirst, 1999), augments in the absolute value of spectroscopic signal of the protein in the presence of certain urea concentrations were, at a first sight, taken to reflect a minor raise in the fraction of the backbone peptide groups involved in regular structural elements promoted by the chaotrope. In other words, it is tempting to assume that urea at 3.00, 7.00 and 8.00 M favoured conformational states of porcine pepsin A with improved order in terms of secondary structure.

A.2. *Tertiary Structure*

With the purpose of furnishing deeper insight into chaotrope-mediated conformational changes of porcine pepsin A, one relied on near-UV circular dichroism as a probe for seeking alterations in terms of integrity of the tertiary structure. Data obtained in the presence of each perturbant are separately summarized in the Figures 4.24 and 4.25.

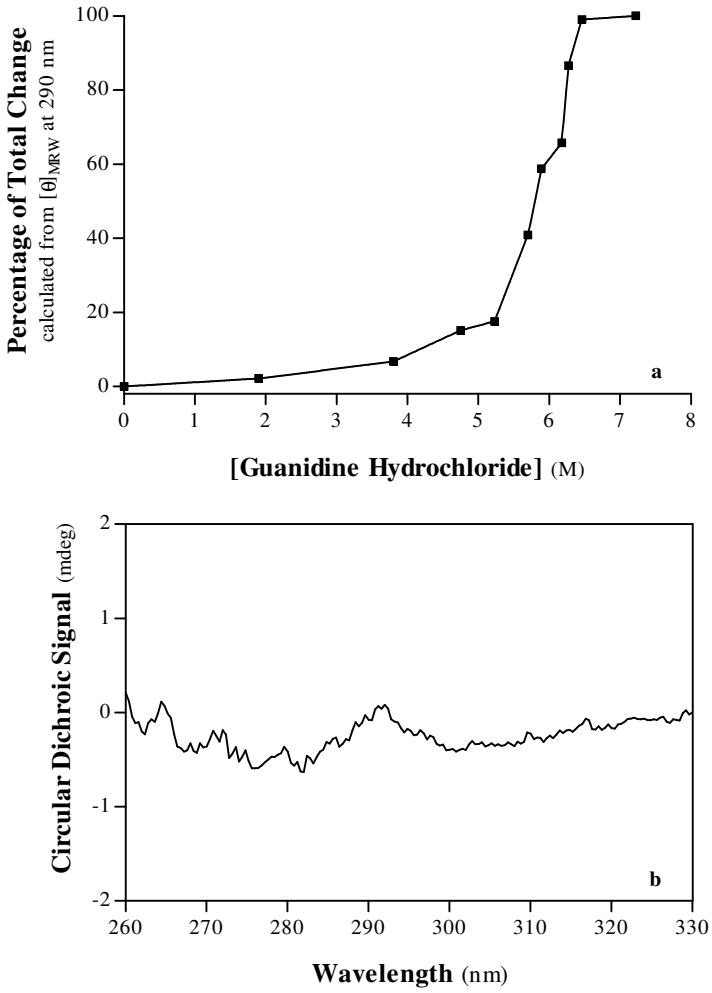

Figure 4.24 – **(a)** Modifications in the mean residue ellipticity of porcine pepsin A at 290 nm induced by guanidine hydrochloride shown as percentage of the total change occurring between 0 and 7.22 M of denaturant. Data were obtained from near-UV circular dichroic spectra, which were recorded after 1 h of exposure of porcine pepsin A to increasing concentrations of the chaotrope at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. **(b)** Raw near-UV circular dichroic spectrum of porcine pepsin A collected after 1 h - incubation at 25ºC in 7.22 M guanidine hydrochloride.

A small set of spectra concerning the impact of guanidine hydrochloride on the tertiary arrangement of the protein under study were generated in a *Jasco J-600* spectropolarimeter, whereas the majority of the spectra obtained during the experiments described in the present dissertation were recorded in a *Jasco J-810* spectropolarimeter. Control samples exhibited different circular dichroic absorbance at 290 nm when near-UV circular dichroic spectra were collected in the two instruments. This discrepancy was assigned to differences between the spectropolarimeters. Consequently, modifications in mean residue ellipticity at 290 nm are shown as percentage of the total change occurring between 0 and 7.22 M guanidine hydrochloride.

As can be seen in Figure 4.24(a), addition of guanidine hydrochloride to the protein-solvent system at concentrations equal to 1.90 and 3.80 M exerted only a negligible effect on the local surroundings of

tryptophan residues. On the contrary, aromatic side chains became unarguably more mobile on going from 3.80 to 5.23 M guanidine hydrochloride. Nevertheless, it is claimed that gross conformational modifications did only take place on adding denaturing salt at concentrations higher than 5.23 M, in view of a larger mean slope of the curve region 5.23 – 6.46 M in comparison with that of the curve region 3.80 – 5.23 M. A general decline in the magnitude of aromatic bands in the wavelength range from 260 to 300 nm reflected severe unpacking of aromatic side chains, which might be symptomatic of drastic weakening and / or loss of tertiary interactions during incubation of porcine pepsin A in concentrations of guanidine hydrochloride up to 6.46 M. On balance, guanidine hydrochloride - induced unfolding of porcine pepsin A as tracked by near-UV circular dichroism apparently corresponded to a multi-step transition. The lower slope of the curve region 3.80 – 5.23 M (in comparison with the curve region resultant from dominant denaturation) conveyed a slower attainment of equilibrium. However, if one overlooked the apparent multiphasic nature of the denaturation transition, an attempt to fit a sigmoidal model to the curve in the Figure 4.24(a) (with an R^2 equal to 0.98) should yield a transition midpoint concentration of 5.87 ± 0.082 M, which is in excellent agreement with that estimated for the denaturation transition followed by far-UV circular dichroism.

From the curve in the Figure 4.24(a), it is clear that a final guanidine hydrochloride-denatured state was sampled by porcine pepsin A at chaotrope concentrations equal to and higher than 6.46 M. Judging by the proximity of the circular dichroic signal to zero, along with the absence of typical aromatic bands [see Figure 4.24(b)], the end-point of the chaotrope-mediated denaturation transition consisted of a disordered conformational state, whose aromatic side chains no longer occupied fixed positions. Nonetheless, a small peak centred at about 292 nm in the raw near-UV circular dichroic spectrum of porcine pepsin A generated after 1 h - incubation in 7.22 M guanidine hydrochloride might be reminiscent of the fine peak at 290 – 292 nm observed in the near-UV circular dichroic spectra of native porcine pepsin A (see Figure 4.5). This observation is plausibly explained by residual entrapment of one or a few tryptophan side chains within residual tertiary structure. The supposition for local conservation of tertiary arrangements is in harmony with the presence of three disulphide bridges in non-reduced porcine pepsin A, and it has already been hypothesized in the scope of acetonitrile-induced denaturation of porcine pepsin A (*vide* subsection 1.A.2. '*Tertiary Structure*').

As for the case of urea, it is clear that its deleterious impact on the circular dichroic absorbance in the near-UV region shown by porcine pepsin A was considerably smaller as compared to that of guanidine hydrochloride. This conclusion is withstood by experimental data in the Figure 4.25.

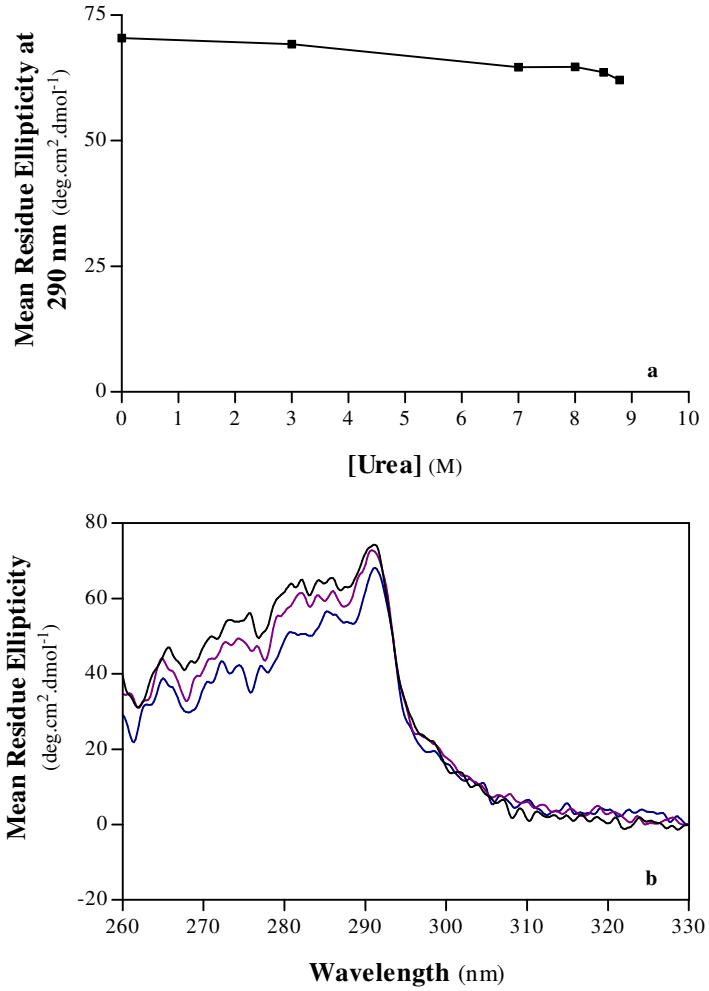

Figure 4.25 – **(a)** Dependence of the mean residue ellipticity of porcine pepsin A at 290 nm on urea molarity. The line was merely used to connect the symbols. Data were obtained from near-UV circular dichroic spectra, which were recorded after 1 h of exposure of porcine pepsin A to increasing concentrations of the chaotrope at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. **(b)** Near-UV circular dichroic spectra of porcine pepsin A collected after 1 h - incubation at 25ºC in the absence (___), and in the presence of 3.00 M (___) and 8.79 M (___) urea.

In contrast to the trend observed for the secondary structure, exposure of porcine pepsin A to varying concentrations of urea for 1 h at 25ºC brought about minor drops in the mean residue ellipticity at 290 nm [see Figure 4.25(a)]. The largest reduction in the magnitude of aromatic bands was detected at the highest urea concentration tested, 8.79 M. It was remarkable to note that the mean residue ellipticity value, for instance, at 290 nm shown by porcine pepsin A in the presence of this urea concentration corresponded to as much as 88.16% of the native value. In addition, spectral shapes obtained in the aromatic region akin to those of the well-folded conformational state were maintained, irrespective of the urea concentration tested [see Figure 4.25(b)]. The underlying conclusion from this analysis is that the aromatic side chains might have become somewhat further apart, and their local surroundings might have become slightly less ordered.

Nonetheless, there were aromatic chromophores still confined in hydrophobic cores, on account of preservation of considerable tertiary packing upon exposure for 1 h to urea at any of the examined concentrations.

In order to complement information described in the previous paragraphs, concerning the influence of chaotropes on the tertiary structure of porcine pepsin A, intrinsic fluorescence measurements were performed, in which tryptophan fluorophores were used as probes of microenvironmental polarity. Spectra were examined in terms of wavelength of maximum fluorescence emission. Results obtained are summarized in the Figure 4.26 and, in general, corroborate with the conclusions drawn from near-UV circular dichroism experiments.

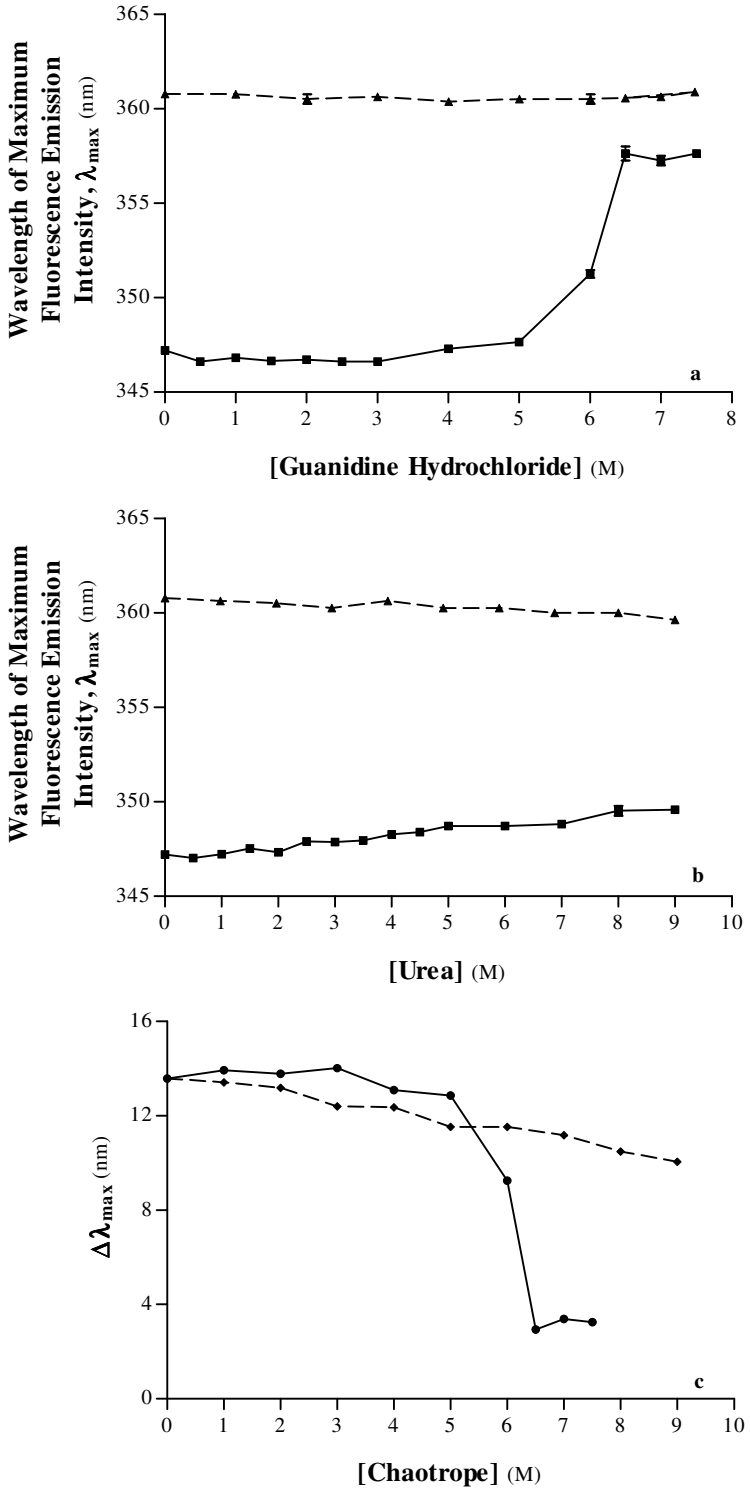

Figure 4.26 – (a, b) Changes in the wavelength of maximum fluorescence emission by NATA (triangles; _ _) and porcine pepsin A (squares; ___) as a function of guanidine hydrochloride **(a)** and urea **(b)** concentration. **(c)** Effect of increasing concentrations of guanidine hydrochloride (circles; ___) and urea (diamonds; _ _) on the difference between the wavelengths of maximum fluorescence emission by NATA and porcine pepsin A. Data were taken from intrinsic tryptophanyl fluorescence emission spectra, which were generated after 1 h of exposure of NATA and porcine pepsin A to increasing concentrations of each chaotrope at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot.

As proved by the Figure 4.26(a, b), the position of fluorescence emission spectra of NATA remained essentially constant over the entire ranges of guanidine hydrochloride and urea concentrations spanned. Thereby, changes in fluorescence properties of the protein under study were surmised to be specific effects of the chaotropes, and to originate from conformational alterations in areas near one or more tryptophan residues.

Great disparity between the actions of both chaotropes on porcine pepsin A was detected. In moderate agreement with the picture drawn by circular dichroism measurements in the aromatic region, large-scale denaturation of porcine pepsin A was triggered at concentrations higher than 5.00 M guanidine hydrochloride [see Figure 4.26(a, c)], as reflected by a large red shift in the peak position of fluorescence emission (approximately 9.98 nm). This behaviour is commonly associated with an augment in the polarity of the surrounding environment of tryptophan side chains, which could be caused by disruption of protein hydrophobic cores (as inferred from near-UV circular dichroism data) resulting in the exposure of the aromatic fluorophores to the surface of the protein. If a sigmoidal model was fitted to the curve corresponding to the effect of guanidine hydrochloride in Figure 4.26(c) (with an R^2 equal to 0.99), the denaturant concentration at half-completion of the transition should equal 6.01 M.

The end-point seen at concentrations of guanidine hydrochloride equal to and above 6.50 M corresponded to an expanded macrostate, whose wavelength of maximum fluorescence emission (approximately 357.50 nm) is supportive of greatly exposed tryptophan residues [see Figure 4.26(a)]. Nonetheless, the product of guanidine hydrochloride - induced transition is claimed not to have its tryptophan residues completely solvent-accessible, on the grounds of a $\Delta\lambda_{max}$ value (*i. e.*, the difference between the two emission maxima of NATA and porcine pepsin A) equal to 3.25 nm at the highest chaotrope concentration in the protein-solvent system [see Figure 4.26(c)]. Once more, the picture drawn for the final guanidine hydrochloride - denatured state of porcine pepsin A from intrinsic tryptophanyl fluorescence experiments is in accord with the persistence of local tertiary structure, which was earlier proposed on the basis of evidence from circular dichroism measurements in the aromatic region.

Porcine pepsin A behaved differently when incubated for 1 h in growing concentrations of urea in relation to guanidine hydrochloride. In effect, it was noted a nearly monotonic dependence of the position of tryptophanyl fluorescence emission spectra of porcine pepsin A on urea molarity [see Figure 4.26(b)]. And, only a subtle red spectral shift (equal to about 2.36 nm) was detected on going from 0 to 9.00 M of chaotrope. This is tantamount to saying that tryptophan residues became slightly more exposed to the solvent. However, under the selected experimental conditions, porcine pepsin A at 9.00 M urea was still highly ordered with significantly shielded tryptophan residues, whose delicate environments should be very close to those in the native state. This scenario is confirmed by minute structural changes probed by near-UV circular dichroism measurements.

Finally, it is worth emphasizing that tertiary interactions and microenvironments of tryptophan residues of porcine pepsin A were apparently more affected by urea at concentrations equal to and lower than 5.00 M in comparison with equimolar solutions of guanidine hydrochloride; even though the denaturing efficiency of the latter chaotrope at concentrations above 5.00 M was strikingly higher than that of

corresponding amounts of urea.

A.3. *Interpretative Remarks*

Notorious differences in the impact of guanidine hydrochloride and urea on the native conformational state of porcine pepsin A are testified by the spectroscopic data earlier described in the ongoing section. In the chosen experimental setup, the protein underwent an order-to-disorder transition upon exposure to concentrations of guanidine hydrochloride over the range of 1.90 – 7.50 M for 1 h at 25ºC. Under parallel conditions, the target protein was little affected when it was incubated in aqueous solutions with varying amounts of urea, even though experimental data obtained in this scope illustrate two opposing effects of the non-electrolyte on porcine pepsin A. On one hand, urea induced gentle decay of the tertiary arrangement. On the other hand, urea was noted to promote local backbone order, despite the orthodox conception of this compound as a disordering agent. Opposing solute effects on porcine pepsin A shall be discussed separately.

A.3.1. *Non-Conventional Effects of Urea: Improvement of Backbone Order*

From far-UV circular dichroic spectra, it is apparent an increase in the backbone order on incubating natively folded porcine pepsin A in the presence of 3.00, 7.00 and 8.00 M urea for 1 h [see Figure 4.23(a, c)]; although the tertiary arrangement underwent subtle debilitation as probed by near-UV circular dichroism and intrinsic tryptophanyl fluorescence [see Figures 4.25 and 4.26(b, c)]. Evidence for structure-promoting effects by urea is not completely surprising, for a deep look at open literature shall provide us with a few studies pointing to the ability of this compound to induce formation of helical structure in short peptides and denatured conformations of proteins (Whittington, *et al.*, 2005), as well as marked increases in overall secondary structure of protein folded states (Gull, *et al.*, 2006a; Gull, *et al.*, 2006b). Nonetheless, this is so far, to the author's knowledge, the first report on urea-assisted augment in ordered structure of porcine pepsin A. Moreover, analogous behaviour of the backbone of other folded proteins in response to urea has been reported only at low denaturant concentrations (Gull, *et al.*, 2006a; Gull, *et al.*, 2006b). Thus, the raise in ordered backbone structure in folded porcine pepsin A after exposure to near-saturating concentrations of urea for 1 h should also be stressed.

Why urea at low concentrations acts as a protein structure-promoter is not very clear at present. Herein, it is hypothesized that the enhancement of structureness in porcine pepsin A was a small-scale phenomenon instigated by favourable interaction of urea with the polypeptide backbone. Whittington and collaborators (2005) have interpreted urea-mediated formation of helical structure by surmising that the backbone becomes rigid upon interaction with the neutral compound. Loop regions located at the protein surface and, hence, solvent-accessible (Berg, *et al.*, 2002; Cooper, 2000b) might be considered potential

targets of urea action in this scope. Given that main chain α-carbonyl and α-amide groups of loops are not hydrogen-bonded to each other, these polypeptide segments usually display irregular shapes and tend to be more flexible than helices or β-sheets (Branden & Tooze, 1999).

A.3.2. *Conventional Effects of Chaotropes: Induction of Unfolding*

A.3.2.1. *Guanidine Hydrochloride*

In respect of guanidine hydrochloride - mediated unfolding of porcine pepsin A, gross conformational changes were triggered at 4.75 – 5.23 M of chaotrope [see Figures 4.23(a), 4.24(a) and 4.26(a, c)]. Comparative analysis of denaturation curves obtained resorting to different spectroscopic probes hints at a multi-step unfolding transition (see Figure 4.27).

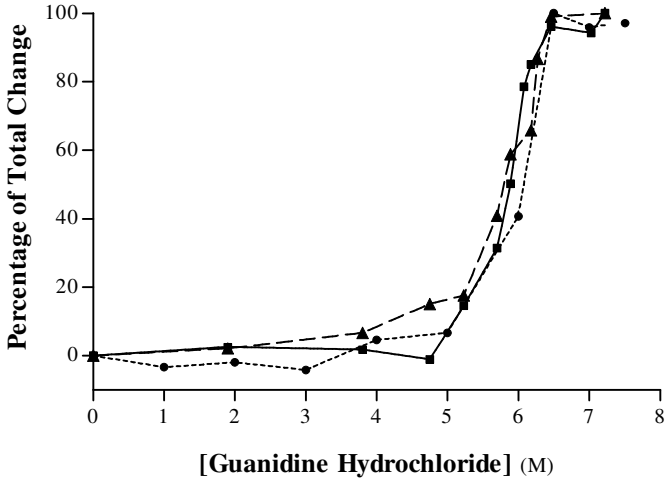

Figure 4.27 – Modifications in structural properties of porcine pepsin A upon exposure to growing concentrations of guanidine hydrochloride for 1 h and 25ºC. Data are represented as percentage of the total change in the mean residue ellipticity of porcine pepsin A at 216 nm (squares; ___) and 290 nm (triangles; _ _), and in the difference between wavelengths of maximum fluorescence emission by NATA and the protein (circles; …..), occurring over the range of denaturant concentration spanned. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot.

Indeed, non-coincidence of profiles of changes in secondary and tertiary structures indicates that the unfolding reaction induced by the charged denaturant might have been non-cooperative and proceeded with the accumulation of stable intermediate(s). With these data, however, it is not possible to ascertain the nature of the, so far, hypothetical intermediary conformational state(s). Assays described elsewhere succeeded in furnishing better insights into the unfolding pathway of porcine pepsin A assisted by guanidine hydrochloride (see section 2.E. '*Induction of Denaturation under Non-Standard Experimental Conditions*').

Under the specific conditions used in this research, the final conformational state of guanidine

hydrochloride - denatured porcine pepsin A, which was detected at chaotrope concentrations equal to and higher than about 6.50 M, was incompletely unfolded. It contained a considerable amount of ordered elements of secondary structure [see Figure 4.23(a, b)]. And, although native hydrophobic cores were almost completely disrupted, as judged from very loose packing and high exposure of tryptophan residues [see Figures 4.24 and 4.26(a, c)], it is speculated that local tertiary structure was preserved.

According to Schellman (2003), the dominant denaturant effect of guanidine hydrochloride stems from the guanidinium ion, even though the chloride ion should not be considered as negligible. Most certainly, the negative electrostatic potential at the surface of the natively ordered state of porcine pepsin A hampered approaching of anions. Therefore, the tentative structural interpretation of the effect of guanidinium hydrochloride on the protein under study presented in the current dissertation will solely focus on the action of the cation.

Before proceeding, one shall look at the structure and hydration of guanidinium ion. It is a highly symmetrical and rigid ion with relatively low charge density. Owing to the linearity of hydrogen bonds established between the guanidinium ion and water molecules, the latter are strongly constrained to remain within the molecular plane of the cation. The guanidinium ion forms only three undistorted hydrogen bonds with water molecules, which are weaker and less structured than hydrogen bonds observed in pure water. Furthermore, there is incompatibility between the bond angle of H – N – H (the local structure being hydrated) and the short-range tetrahedral structure of water. The characteristics listed above (pertinent to the guanidinium ion and the intercomponent hydrogen bonds) account for the weak hydration of the cation (Mason, *et al.*, 2003; Mason, *et al.*, 2004). Indeed, Mason and collaborators (2003) have demonstrated that the guanidinium ion does not efficiently compete for hydrogen bonds within the water network. And, data obtained by Courtenay and co-workers (2001) have evidenced stronger favourable self-interactions of denaturant than interactions of the solute with water.

Correlations have been drawn between poor hydration of the guanidinium ion and its denaturing properties. A practically unrecognizable hydration shell lends support to the postulate, according to which preferential binding of the guanidinium ion to the protein surface provides a noteworthy contribution to the disordering action of guanidine hydrochloride (Mason, *et al.*, 2003). It is well-established that the primary target for the action of the guanidinium ion is the polar main chain surface (Courtenay, *et al.*, 2001; Möglich, *et al.*, 2005; Robinson & Jencks, 1963). The cation is capable of interacting with other protein groups, but to a lower extent as compared to peptide groups. Accumulation of the guanidinium ion near negatively charged surface is taken to be moderate, while it exhibits only random distribution near non-polar and polar-neutral side chain groups (Courtenay, *et al.*, 2001). It is of relevance to add that guanidinium ion (like urea) interacts with main chain and polar side chain groups via multiple hydrogen bonds, and with non-polar groups via van der Waals interactions (Caballero-Herrera, *et al.*, 2005; Del Vecchio, *et al.*, 2002; Dunbar, *et al.*, 1997; Pike & Acharya, 1994; Zou, *et al.*, 1998).

In consonance with what was stated above, direct interaction of guanidinium ions with the backbone of porcine pepsin A is presumed to have given a key contribution to stabilization of the denatured state. The predominant entity newly exposed to solvent during protein unfolding are main chain groups (Timasheff &

Xie, 2003), which become available for denaturant binding. Peptide groups are mostly involved in the formation of regular elements of secondary structure (Fersht, 1999; Lesk, 2004). And, hence, hydrogen bonds established between main chain groups are very important from the viewpoint of protein structure maintenance. In view of this, exposure of backbone, hitherto buried within the folded structure, and competition of guanidinium ions for intramolecular hydrogen bonds might partially explain the looseness in the secondary structure detected above 4.75 M guanidine hydrochloride.

The hydrophobic effect is well-established to give a critical contribution to protein structuring and conformational stability (Chan & Dill, 1990; Finney, *et al.*, 1993). Its impairment may be caused by deformation of hydration structures at protein surface, resulting from modification in the arrangement and properties of water. Reduced local water density is a second consequence following from weak hydration of the guanidinium ion (Mason, *et al.*, 2003). And, such a change in the hydrogen-bonded network of water is believed to reduce hydrophobic hydration by displacing water molecules from the solvation sphere of non-polar entities (Mason, *et al.*, 2003; Muller, 1990). Therefore, the penalty for exposure of non-polar groups to solvent is lowered in comparison with pure water (Breslow & Guo, 1990; Nozaki & Tanford, 1970; Vanzi, *et al.*, 1998). Ability of the guanidinium ion to interact with hydrophobic surfaces (Mason, *et al.*, 2004; Washabaugh & Collins, 1986) may further perturb the hydrophobic effect. Thus, stabilization of disordered relative to native conformations of porcine pepsin A mediated by guanidinium ion might have been fundamentally driven by a more prevalent association with expanded forms, and an increased solubility of the non-polar side chains buried in the cores of the folded protein as result of disorder of the water arrangement by the cation.

Comparison of denaturation curves obtained by measurements of far- and near-UV circular dichroism after a 1 h - incubation in aqueous solutions of denaturing salt revealed that modest disturbance in the protein tertiary packing occurred before gross unfolding (see Figure 4.27). In view of this, one can speculate that a priming event in guanidine hydrochloride - induced unfolding of porcine pepsin A consisted of some loss of compactness caused by changes in the solvation shell and ensuing increase, to some extent, in solubility of non-polar side chains. This phenomenon could have facilitated the access of solvent to the hydrophobic cores, which contributed to the onset of dominant unfolding at both levels of protein structure organizational hierarchy, the secondary and tertiary structures.

A.3.2.2. *Urea*

As aforementioned, the backbone order of porcine pepsin A was incremented by urea at 3.00, 7.00 and 8.00 M, and remained essentially unaffected in the presence of higher molarities of denaturant. By contrast, the tertiary structure was slightly disordered by the denaturant at every molarities tested [see Figures 4.25 and 4.26(b, c)]. Nonetheless, even on exposing porcine pepsin A to near-saturating urea concentrations, the protein still adopted highly folded conformations. Even though aromatic side chains might have become slightly more mobile and less shielded from the solvent (in particular, tryptophan side chains), their arrangement was still considerably ordered and constrained.

Recalling the dependence of structural properties of porcine pepsin A on guanidine hydrochloride molarity, one can conclude that there was a striking difference in the efficiency of the two denaturants for disturbing tertiary interactions and packing. Aqueous solutions of guanidine hydrochloride at transitional and post-transitional concentrations brought about greater degrees of structural disarray in porcine pepsin A than equimolar aqueous solutions of urea (see Figure 4.26). This is not unexpected taking into account that, with only a few exceptions (Greene & Pace, 1974; Makhatadze, *et al.*, 1998), the charged denaturant has been deemed about (2 – 2.5) – fold more effective in inducing unfolding of monomeric proteins than the neutral denaturant (Myers, *et al.*, 1995; Pace, 1986; Smith & Scholtz, 1996). Dissimilarities in the denaturing potentials of guanidine hydrochloride and urea stem from a summation of factors (Courtenay, *et al.*, 2001; Del Vecchio, *et al.*, 2002; Dempsey, *et al.*, 2005; Schellman, 2003), such as larger preferential solvation of the overall protein surface by the guanidinium ion (Courtenay, *et al.*, 2001; Schellman, 2003). Greater interaction of urea molecules *vis-à-vis* guanidinium ions with water (Courtenay, *et al.*, 2001; Soper, *et al.*, 2003) might, at least, partially rationalize different partitioning of the denaturants between the bulk solution and the protein surface. Backbone groups are considered primary regions of interaction of urea, analogously to the guanidinium ion (Scholtz, *et al.*, 1995; Timasheff & Xie, 2003). And, it is only randomly distributed in the vicinity of polar-charged, polar-neutral and non-polar side chain groups (Courtenay, *et al.*, 2001).

Available data do not allow a clear discrimination between the two types of molecular mechanisms of the denaturants action and, thus, the relative importance of each of them in causing the observed effects of urea on the tertiary structure of porcine pepsin A remains ambiguous. Nevertheless, by means of molecular dynamics simulations, Bennion and Daggett (2003) have shown that direct interaction of urea with chymotrypsin inhibitor became particularly significant only after disruption of the secondary structure. On this basis, the minute impact of urea on the tertiary structure of porcine pepsin A is interpreted mainly by surmising that the chaotrope was able to affect the structure of the hydrogen-bonded network of water and, thus, solvent-mediated hydrophobic interactions.

Demonstrations of substantial hydrogen-bonding capacity of the non-electrolyte and of its easy incorporation into the water network are compatible with a marked effect of urea on the three-dimensional network of water (Soper, *et al.*, 2003). Reports of hydrogen bonds between water molecules in the solvation sphere of the carbonyl oxygen atom of urea molecules more strained as compared to those in bulk solvent (Vanzi, *et al.*, 1998), and of a lower ratio of the number of short hydrogen bonds (≤ 1.8 Å) to the number of standard hydrogen bonds (2.4 Å) between water molecules in the presence of urea (Bennion & Daggett, 2003) substantiate the potential of this compound to perturb the ability of water to maintain its hydrogen-bonding pattern, thereby weakening the water structure. Some authors have inclusively described the displacement of water molecules from solvent structures surrounding non-polar entities by urea molecules, similarly to what has been reported for the guanidinium ion (Caballero-Herrera, *et al.*, 2005; Muller, 1990; Soper, *et al.*, 2003).

At high molarities of urea, solute-induced perturbations of water are known to be magnified (Bennion & Daggett, 2003). In the case of porcine pepsin A, however, aqueous solutions of the chaotrope at near-saturating concentrations were hardly able to alter protein packing. This finding might suggest that the structure of porcine pepsin A is maintained by a strong hydrophobic effect.

Other details on the behaviour of porcine pepsin A in the presence of guanidine hydrochloride and urea will be given under the heading '*Induction of Denaturation under Non-Standard Experimental Conditions'*.

B. *Hydrodynamic Behaviour*

Variations in the hydrodynamic dimension of porcine pepsin A under denaturant effect were surveyed resorting to size exclusion chromatography. As reported earlier, there was substantial adsorption of porcine pepsin A to the matrix under native-like conditions and in the presence of low concentrations of acetonitrile. Interaction of the protein with the stationary phase was claimed to be hydrophobic in nature (see section 1.B. '*Hydrodynamic Behaviour'*). A similar behaviour was observed whenever the incubation medium and the mobile phase contained small amounts of both employed chaotropes, thereby hampering the straightforward establishment of a correlation between changes in the elution position of porcine pepsin A and intrinsic perturbations induced by guanidine hydrochloride and urea at concentrations up to 1.50 and 2.50 M, respectively. For instance, whenever porcine pepsin A was exposed to and run in the presence of 0.50 and 1.00 M of any of the denaturants, the protein eluted later than expected and as a broad and markedly tailing peak. Interaction of porcine pepsin A with the stationary phase was deemed to be negligible and not to influence the protein elution behaviour, only when the solvent conditions (during the incubation phase and the chromatographic analysis) were of such a kind that the following requisites were verified (for each set of chromatograms pertaining to each chaotropic agent): (i) similar peak areas; (ii) similar peak shapes (symmetrical or nearly symmetrical); and (iii) minimal amounts of protein eluted during washing steps. In view of this, the elution volume was surmised to be solely dependent on the protein Stokes radius when the concentration of guanidine hydrochloride and urea equalled or exceeded 2.39 and 3.00 M, respectively. Size exclusion chromatographic patterns of porcine pepsin A previously incubated and analyzed in the presence of varying concentrations of each chaotrope are depicted in the Figure 4.28(a, c).

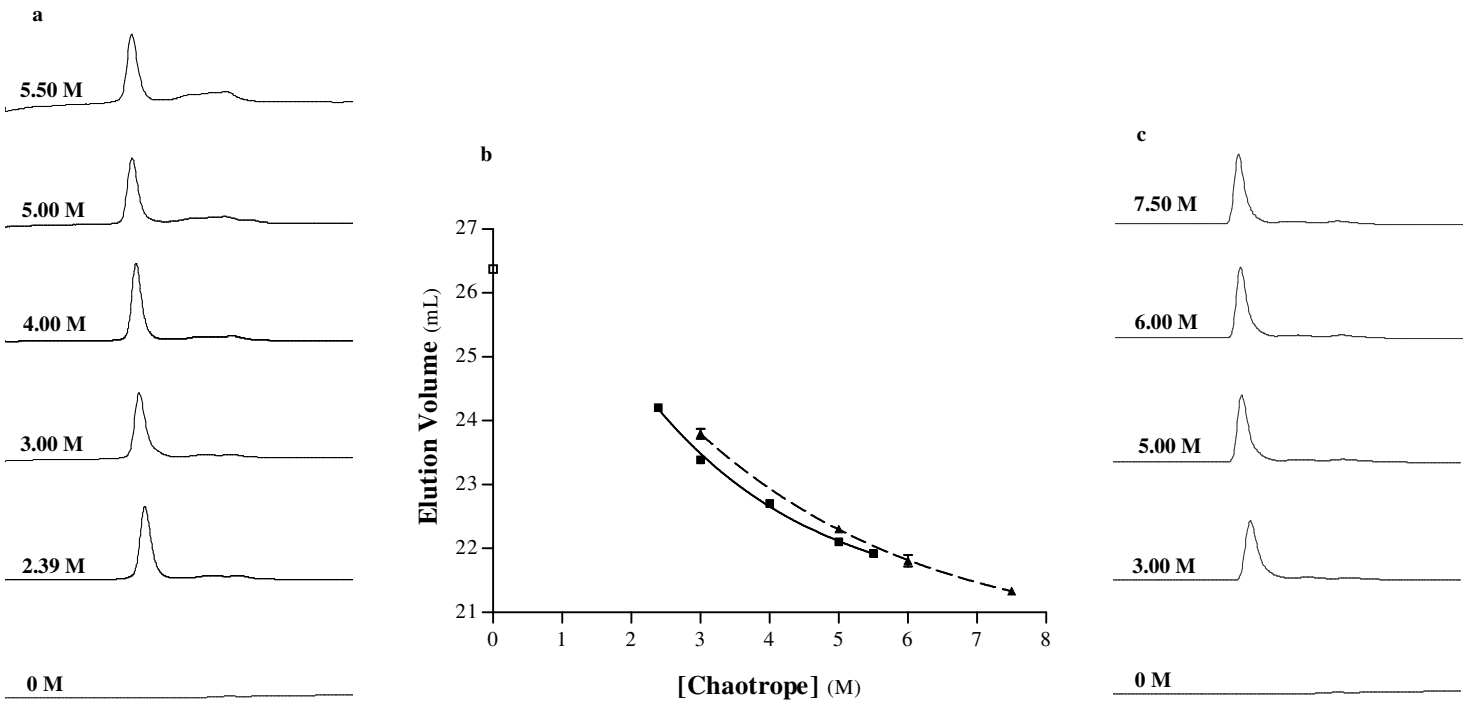

Figure 4.28 – **(a, c)** Size exclusion chromatographic profiles for porcine pepsin A in a *Superdex 75 HR 10/30* column after 1 h - incubation in growing concentrations of guanidine hydrochloride **(a)** and urea **(c)** at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. **(b)** Influence of guanidine hydrochloride (squares) and urea (triangles) concentration on the elution volume of porcine pepsin A. Non-linear regression analysis of the experimental data obtained in the presence of guanidine hydrochloride (___) and urea (_ _) yielded an R^2 equal to 0.99. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot. The empty square stands for the theoretical elution volume of porcine pepsin A in aqueous medium calculated from the calibration curve.

In the experimental setup used for the research conducive to this dissertation, decreasing trends were observed for the variation of the elution position for porcine pepsin A as a function of guanidine hydrochloride and urea concentrations [see Figure 4.28(b)]. Furthermore, high proximity between elution volumes for the protein under the effect of equal concentrations of both chaotropic agents was noted, with slightly higher values obtained in the presence of urea. On balance, information retrieved from Figure 4.28 hint at a raise in the Stokes radius of porcine pepsin A upon submission to guanidine hydrochloride (2.39 – 5.50 M) and urea (3.00 – 7.00 M) concentrations which do not induce large-scale conformational perturbations, as proved in the foregoing section.

In the transition from 5.00 to 5.50 M guanidine hydrochloride, a drop in peak area was detected [see Figure 4.28(a)]. This fact is rationalized by the appearance of a short and excessively broad peak posterior to the main peak. As evidenced by spectroscopic data, drastic disordering of porcine pepsin A was triggered at around 5.00 M guanidine hydrochloride. Such reaction is generally accompanied by transfer of some buried non-polar groups to the protein surface, which should propitiate new conditions favourable for protein adsorption to the matrix through hydrophobic interactions. This proposal is substantiated by strong retention of porcine pepsin A in the chromatographic column under other denaturing conditions, such as neutral-alkaline pH, as it shall be sketched in the following paragraph.

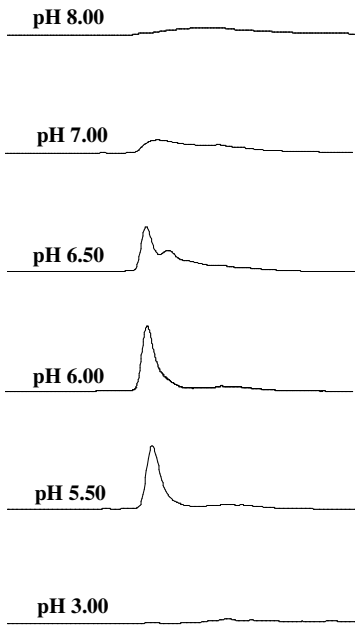

Figure 4.29 – Size exclusion chromatographic patterns of porcine pepsin A in a *Superdex 75 HR 10/30* column after incubation in phosphate-citrate buffers, titrated to varying pH values, for 1 h at 25ºC.

Size exclusion chromatographic analyses of porcine pepsin A at varying pH values were performed in the same column and under parallel conditions as those provided in the remaining assays (see Figure 4.29). Maintenance of buffering capacity in the pH range from 3 to 8 demanded the use of phosphate-citrate buffers as running and sample buffers (instead of 10 mM sodium acetate solutions). As such, after 1 h - incubation of porcine pepsin A in a phosphate-citrate buffer titrated to a specific pH value at 25ºC, the protein was applied to the column and eluted with a matching buffer. Contrary to the behaviour reported for porcine pepsin A at pH 3, protein binding to the matrix was minimized at pH 5.5 and 6. Mean elution volumes calculated from size exclusion chromatographic profiles corresponding to protein incubated in phosphate-citrate buffers at pH 5.5 and 6 for 1 h were 25.12 and 24.62 mL, respectively. These values are lower than the one predicted for native porcine pepsin A (26.37 mL). Hence, under the selected experimental conditions, there was a raise in the hydrodynamic radius of the protein on going from pH 3

to 5.5 or 6.

Diverse studies performed under equilibrium and non-equilibrium conditions have consensually indicated the onset of a conformational transition between pH 6/6.5 and 7 (Campos & Sancho, 2003; Kamatari, *et al.*, 2003; Konno, *et al.*, 2000). Above pH 7, pepsin undergoes disruption of secondary structural elements, loss of tertiary interactions and molecular extension (Aoki, *et al.*, 1997; Campos & Sancho, 2003; Dee, *et al.*, 2006; Konno, *et al.*, 2000). It is envisioned that alkaline denaturation should bring about a net reduction in the charge density (Mazzini, *et al.*, 1997), together with an increment in the hydrophobic surface area, which should have accounted for the elution profiles obtained at pH values equal to or higher than 6.5 (this is, partial elution of porcine pepsin A by phosphate-citrate buffer at pH 6.5 as a broad and asymmetrical peak, and nearly full retention of the protein in the column at pH 7 and 8). Although adsorption of partially unfolded conformations to the matrix might well support the chromatographic patterns observed for porcine pepsin A at 5.50 M guanidine hydrochloride, autolysis should not be completely ruled out. Indeed, guanidine hydrochloride and urea were reported to favour autolysis of porcine pepsin A at acidic pH, even though this behaviour was noted to become more relevant at temperature values higher than 25ºC (Blumenfeld, *et al.*, 1960; Khan & Salahuddin, 1977 as quoted by Pain, *et al.*, 1985b; Perlmann, 1959).

Due to limitations in chemical stability of the support medium, effects of guanidine hydrochloride and urea concentrations higher than, respectively, 5.50 and 7.50 M on the hydrodynamic behaviour of porcine pepsin A were not examined. It is worth adding that small differences in average peak areas were detected amongst sets of chromatographic profiles concerning the effect of the various denaturing factors employed. These differences were assigned to slightly dissimilar abilities of each factor to minimize protein-matrix interaction.

B.1. *Interpretative Remarks*

In summary, there was an increase in the Stokes radius on exposing porcine pepsin A to varying guanidine hydrochloride (2.39 – 5.50 M) and urea (3.00 – 7.00 M) concentrations for 1 h at 25ºC; although spectroscopic data do not show marked structural perturbation of the protein under these solvent conditions. Indeed, large-scale unfolding did only occur above 4.75 – 5.13 M of the charged denaturant (see Figure 4.27). And, improvement of local backbone order was found at 3.00 and 7.00 M of the neutral denaturant (see Figure 4.23). Nonetheless, positive changes in the hydrodynamic radius of porcine pepsin A appear to be corroborated by evidence on small disruption of the tertiary structure (see Figures 4.24, 4.25 and 4.26). Therefore, modifications in the hydrodynamic behaviour of native porcine pepsin A over the ranges of chaotrope concentrations spanned here were taken to reflect small-scale swelling and / or unfolding of the molecule.

C. *Thermal Stability*

On measuring heat absorption by pepsin-containing solutions as they were heated, the influence exerted by growing concentrations of guanidine hydrochloride and urea on the protein could be accessed in terms of thermal stability. Regardless of the solvent conditions, each differential scanning calorimetric profile showed a single endothermic peak. Chaotrope-induced deviations of the temperature value at the maximum of the endotherm corresponding to denaturation of native porcine pepsin A are reported in the Figure 4.30.

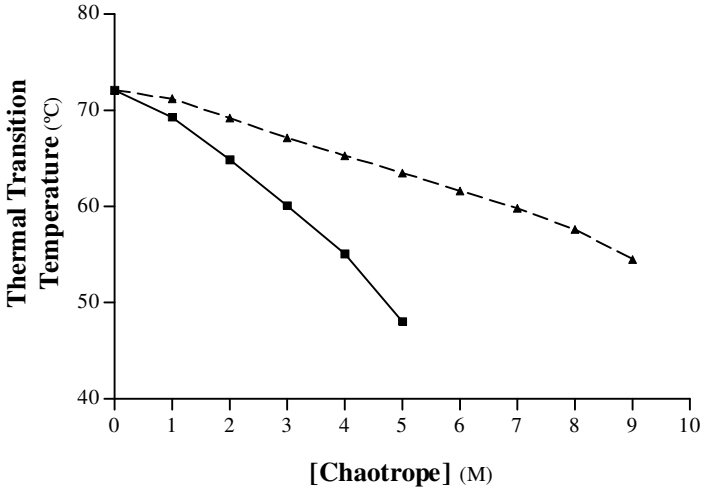

Figure 4.30 – Variations in thermal transition temperature of porcine pepsin A provoked by guanidine hydrochloride (squares; __) and urea (triangles; _ _). Data were acquired from differential scanning calorimetric analyses of the protein after exposure to increasing concentrations of chaotrope during 1 h at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot.

With a raise in the concentration of both chaotropes in the mixed solvent system, the thermal transition temperature was lessened (see Figure 4.30), indicating a decline in the thermostability of porcine pepsin A. It is worth highlighting that decreases were notably sharper as regards guanidine hydrochloride in comparison to urea, insomuch as the thermal transition temperature value attained at 5.00 M of the former perturbant (48.05ºC) was slightly lower than that obtained at 9.00 M of the latter (54.51ºC). Moreover, no endothermic peak was identified (at least, over the temperature interval covered) on examining samples of porcine pepsin A previously submitted to 6.00 M guanidine hydrochloride for 1 h; whilst thermal transitions were detected in the presence of urea concentrations ranging from 1.00 to 9.00 M.

As described in the previous subchapter (1. '*Acetonitrile-Induced Denaturation of Porcine Pepsin A*'), reference experiments using native porcine pepsin A confirmed the irreversible character of the protein thermal denaturation in aqueous medium at pH 3. Phenomena accountable for irreversibility were not, however, perceptible from the shape of the thermograms generated on scanning upward in temperature for

the first time. Analogously, no endotherm was observed after rescanning protein samples containing 2.00 M guanidine hydrochloride; notwithstanding no exothermic effects [very often visible on differential scanning calorimetric profiles for proteins which are irreversibly denatured (Leharne & Chowdhry, 1998)] were clearly recognized in the post-transitional baselines of excess heat capacity curves resulting from a first heating of porcine pepsin A dissolved in aqueous solutions of guanidine hydrochloride. Furthermore, the cooperativity index ($\Delta H_{cal}/\Delta H_{vH}$) was noted to change with concentration of guanidine hydrochloride. Even though van't Hoff enthalpy values derived from calorimetric curves should only be considered apparent, as a result of the irreversible behaviour of thermal denaturation (Tello-Solís & Hernandez-Arana, 1995); good correspondences were still found between the calorimetric and the van't Hoff enthalpy values estimated for the heat-induced transitions of porcine pepsin A at low chaotrope concentrations. In fact, the calorimetric enthalpy to apparent van't Hoff enthalpy ratio equalled about 0.956 and 0.924 for thermal order-to-disorder transition of porcine pepsin A at, respectively, 1.00 and 2.00 M guanidine hydrochloride. These values might suggest that the transition still exhibited a considerable degree of cooperativity in the presence of these denaturant concentrations. However, this ratio dropped markedly on increasing denaturant concentration, insomuch as a van't Hoff enthalpy value better than 2.5-fold greater than the calorimetric enthalpy value was observed at 5.00 M guanidine hydrochloride. One plausible explanation for values of cooperativity index lower than 1 is related to the contributing enthalpies associated with protein aggregation, which was earlier pinpointed as one major competition to the recovery from thermal denaturation.

In respect of heat-induced disordering transitions of porcine pepsin A dissolved in aqueous solutions of urea, high-temperature conformers clearly underwent exothermic intermolecular association, which was recognizable by 'negative' peaks or false 'positive' peaks after the endotherms. In consequence of possible distortion of endothermic peaks by post-transitional events, calculations of the cooperativity index of thermal transitions in the presence of urea were anticipated to be unreliable. A second heating of samples of porcine pepsin A containing urea at 1.00 and 6.00 M yielded no transition peak. It was, thus, concluded that thermal denaturation of porcine pepsin A was irreversible in these solvent conditions.

Similarly to the survey of changes in the hydrodynamic behaviour, effects of chaotropes on thermal stability of porcine pepsin A were examined in parallel with the effect of pH on this biophysical property. Data obtained from differential scanning calorimetric analyses of samples of porcine pepsin A in phosphate-citrate buffers at different pH values, after an incubation step of 1 h at 25ºC, are summarized in the Figure 4.31.

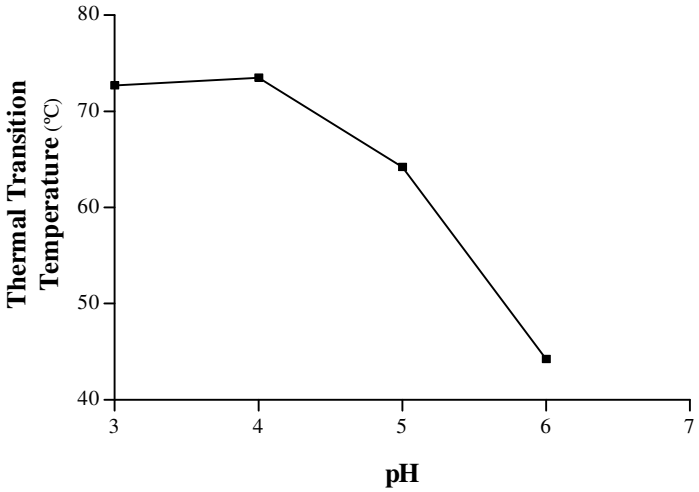

Figure 4.31 – Changes in thermal transition temperature of porcine pepsin A as a function of pH. Data were acquired from differential scanning calorimetric analyses of the protein after incubation in phosphate-citrate buffers, titrated to varying pH values, for 1 h at 25ºC.

A single endothermic peak was observed for every thermogram. Dissimilarities in the nature of salts and ionic strength of aqueous media rationalize minor difference between values of thermal transition temperature determined for porcine pepsin A in 10 mM sodium acetate at pH 3 and in phosphate-citrate buffer at pH 3. Thermal transitions of porcine pepsin A at pH 3 and 4 were completed within similar ranges of temperature. On approaching neutral pH, however, the midpoint temperature of the thermal transition of native porcine pepsin A underwent abrupt decreases, attaining a value equal to 44.25ºC at pH 6. This value is slightly lower than the one computed from thermograms generated for protein previously exposed to 5.00 M guanidine hydrochloride (48.05ºC). Similarly to porcine pepsin A in 6.00 M of charged denaturant, no thermal transition peak could be identified upon heating protein samples in phosphate-citrate buffer at pH 7, at least, over the temperature interval covered.

C.1. *Interpretative Remarks*

It immediately becomes apparent that thermal stability of native porcine pepsin A decreased after the protein was incubated in aqueous mixtures containing varying amounts of guanidine hydrochloride and urea for 1 h (see Figure 4.30). The propensity of the polypeptide backbone to become more ordered in the presence of 3.00, 7.00 and 8.00 M of urea did not bring about global stabilization. However, destabilization exerted by guanidine hydrochloride on porcine pepsin A was always higher as compared to equimolar urea.

It is interesting to note that no major conformational disorder was detected over the range of denaturant concentrations spanned in the set of differential scanning calorimetric experiments (*i. e.*, 1.00 –

5.00 M guanidine hydrochloride and 1.00 – 9.00 M urea). However, hints for moderate depletion in protein compactness had already been given, once reductions in the degree of tertiary structural organization were detected by spectroscopic tools (see Figures 4.24, 4.25 and 4.26), and molecular expansion was demonstrated by size exclusion chromatographic analyses (see Figure 4.28). In all likelihood, higher thermosensitivity of porcine pepsin A in the presence of chaotropes originated from weakening of protein non-covalent interactions (mostly, tertiary interactions) by these compounds during incubation, so that lower temperature values were required for triggering the thermal transition. In addition, the possibility for a conjugate effect of chaotropes and high temperature during sample scanning should not be excluded.

On appraising the influence of pH on the midpoint temperature of thermal denaturation of porcine pepsin A, one could conclude that thermal stability diminished above pH 4 (see Figure 4.31). Hence, it is obvious that the natively folded state did not remain intact after 1 h - incubation at pH 5; even though drastic unfolding of the polypeptide chain has been deemed to be onset between pH 6/6.5 and 7 (Campos & Sancho, 2003; Kamatari, *et al.*, 2003; Konno, *et al.*, 2000). A raise in pH affects the protonation level and, thus, the charge of side chain groups in proteins. Porcine pepsin A is particularly rich in acidic residues. Therefore, destabilization of the protein at pH 5 and 6 is plausibly explained by occurrence of some repulsive interactions between residues exhibiting alike charges, and ensuing molecular extension. In light of data obtained from size exclusion chromatographic assays, an increase in the hydrodynamic radius of porcine pepsin A at pH 5.5 and 6 had already been asserted.

It should be emphasized that no thermal transitions could be detected on heating samples of porcine pepsin A previously incubated in 10 mM sodium acetate at pH 3 with 6.00 M guanidine hydrochloride or in phosphate-citrate buffer at pH 7 for 1 h. In other words, physical forces that determine protein conformational stability were affected to such an extent, throughout the course of incubation in these solvent conditions prior to heating, that enthalpy changes were no longer measurable. This interpretation is compatible with: (i) severe loss of ordered arrangement of aromatic residues and reduction in secondary structural elements induced by moderate and high amounts of guanidine hydrochloride (see Figure 4.27) and neutral pH (Aoki, *et al.*, 1997; Campos & Sancho, 2003; Kamatari, *et al.*, 2003); and (ii) significant exposure of hydrophobic surfaces as inferred from partial or nearly total adsorption of porcine pepsin A to the matrix of the size exclusion chromatographic column, when the protein was under the effect of 5.50 M guanidine hydrochloride or pH equal to or higher than 6.5 (see section B. '*Hydrodynamic Behaviour*' of the current subchapter).

D. *Peptidolytic Activity*

This section focuses on the impact of different amounts of guanidine hydrochloride and urea on the pepsinolytic activity. Denaturant-induced modifications in the rate of porcine pepsin A - catalyzed hydrolysis of the hexapeptide, H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe, are depicted in the Figure 4.32.

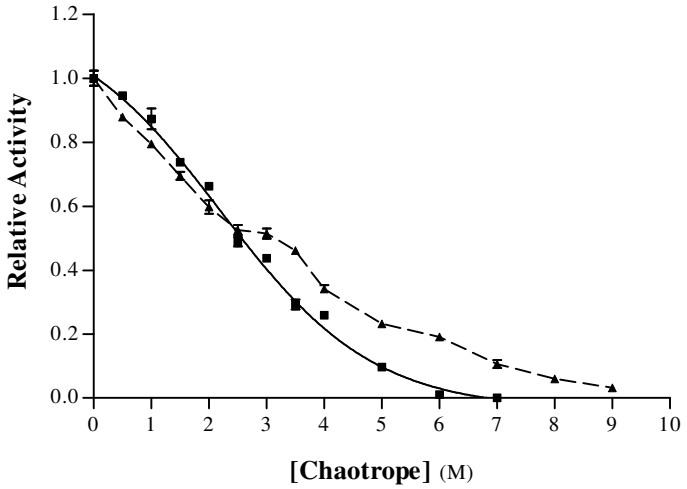

Figure 4.32 – Effect of increasing concentrations of guanidine hydrochloride (squares) and urea (triangles) on the rate of hydrolysis of the synthetic peptide, H – Leu – Ser – *p* – nitro – Phe – Nle – Ala – Leu – OMe, catalyzed by porcine pepsin A at 25ºC. Peptidolytic assays were performed after 1 h - incubation of the enzyme at 25ºC in a mixed solvent system with corresponding chaotrope concentrations. Incubation and reaction media were titrated to pH 3. Non-linear regression analysis of experimental data obtained for the effect of guanidine hydrochloride (___) yielded an R^2 equal to 0.99. With regard to the curve corresponding to the effect of urea, the line (_ _) was merely used to connect the symbols. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot.

In the experimental setup selected for this study, porcine pepsin A retained its capability for catalyzing hydrolysis of the peptide bond *p* – nitro – Phe – Nle over a wide range of concentrations of both chaotropes (see Figure 4.32). However, profiles of peptidolytic activity exhibited by porcine pepsin A under the influence of guanidine hydrochloride and urea conveyed differences between their modes of action. Growing concentrations of guanidine hydrochloride induced a progressive disappearance of enzymatic activity. 50% of maximum activity was lost at 2.22 ± 0.168 M guanidine hydrochloride, and it was completely abolished at 7.00 M of the denaturing salt. On the other hand, decrease in the initial velocity of the hydrolytic reaction on increasing urea concentration revealed to be stepwise, with a noticeable plateau in the interval from 2.50 to about 3.50 M. Residual catalytic activity was still detected after exposure of the enzyme to 9.00 M of this perturbant.

D.1. *Interpretative Remarks*

One conclusion withdrawn from comparison of structural data with information depicted in the Figure 4.32 is that deceleration of the cleavage rate of the peptide bond *p* – nitro – Phe – Nle after incubation of porcine pepsin A for 1 h in guanidine hydrochloride and urea concentrations in the ranges of 0.50 – 4.00 M and 0.50 – 9.00 M, respectively, was not coupled with overall denaturation [see Figures 4.23(a), 4.24(a), 4.25(a) and 4.26]. Similar observations had already been reported for the influence of acetonitrile on

functional properties of porcine pepsin A (see section D. '*Peptidolytic Activity*' of the previous subchapter). The reduction in the pepsinolytic activity over the aforementioned ranges of chaotrope concentrations was, however, accompanied by modest changes in the secondary and / or tertiary structures [see Figures 4.23(a), 4.24(a), 4.25(a) and 4.26], increases in the Stokes radius (see Figure 4.28) and reductions in thermal stability (see Figure 4.30). And, hence, the decrease in the catalytic activity might be plausibly explained by occurrence of local modifications in the active site region and / or 'flap'. As asserted elsewhere, optimal functioning of this enzyme should demand a proper balance between conformational flexibility and rigidity at the catalytic apparatus. Therefore, any minor structural rearrangement capable of affecting such equilibrium might impact the velocity of porcine pepsin A - catalyzed reactions.

Decline in the peptidolytic activity at concentrations equal to or higher than 5.00 M of charged denaturant must have, in large part, originated from major losses of native secondary and tertiary arrangements in the protein as evidenced by spectroscopic data. And, although the final denatured state induced by guanidine hydrochloride exhibited residual structure, it appeared to be inactive, taking into account the absence of hydrolysis at 7.00 M of this denaturant.

The capability of porcine pepsin A to catalyze hydrolysis of the synthetic substrate was found to be largely impaired in highly concentrated solutions of urea, even though the protein preserved a high degree of structural organization. Other mechanisms might well be in operation during enzymatic inactivation, besides local conformational perturbations. Elucidation of this subject requires further investigation. However, it is proposed that urea could, for instance, interfere with the hydration at the active site and vicinal regions, whose significance for catalysis by the enzyme has already been mentioned (see section D. '*Peptidolytic Activity*' of the previous subchapter). The strong hydrogen-bonding ability of urea might enable replacement of water molecules by chaotrope molecules in the microenvironment of the catalytic apparatus. However, the role of water molecules in or nearby the active site and 'flap' might not be entirely fulfilled by the chaotrope. With regard to the stepwise nature of the activity profile in the presence of urea, it still awaits for convincing explanation.

On the whole, porcine pepsin A remained functionally active over a wide range of concentrations of guanidine hydrochloride and urea. This behaviour was, in part, correlated with great resistance of native porcine pepsin A against denaturation by chaotropes, in the experimental conditions used throughout this research. As it shall be seen later, chaotrope-induced conformational perturbations in porcine pepsin A seem to be influenced by other factors, apart from denaturant concentration. Similarly, on surveying literature, one could conclude that the extent to which hydrolytic rates are affected by guanidine hydrochloride and urea depends on the period of exposure to solvent conditions, pH and temperature (Blumenfeld, *et al.*, 1960; Perlmann, 1956).

E. *Induction of Denaturation under Non-Standard Experimental Conditions*

In order to draw a more detailed picture of the response of natively folded porcine pepsin A to the

presence of guanidine hydrochloride and urea, the period of exposure of the target protein to chaotropic agents at 25ºC, prior to collection of structural data, was extended. As such, protein samples containing varying amounts of guanidine hydrochloride or urea, which had been previously examined after a 1 h - incubation, were re-inspected 3 days after their preparation.

Finally, preliminary insights pertinent to the conjunct action of organic solvents and classic chemical denaturants were furnished on evaluating the behaviour of the protein in aqueous mixtures of sodium acetate, acetonitrile and guanidine hydrochloride. These assays did also include a phase of protein incubation in the diverse solvent conditions at 25ºC, which lasted 1 h.

In the denaturation experiments previously outlined, conformational changes in natively ordered porcine pepsin A were tracked by circular dichroism in far- and near-UV regions. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. It is of relevance to remember that glycine was added at a final concentration of 5 mM to incubation media containing urea as mentioned earlier in the section 2.A. '*Preparation of Solutions*' of the chapter III. Control experiments did not return major differences between spectral behaviours of porcine pepsin A in the absence and presence of glycine.

E.1. *Structural Properties*

E.1.1. *Secondary Structure*

Profiles of guanidine hydrochloride - induced denaturation of porcine pepsin A obtained by measurements of circular dichroic absorbance in the amide region after different incubation periods are depicted in the Figure 4.33.

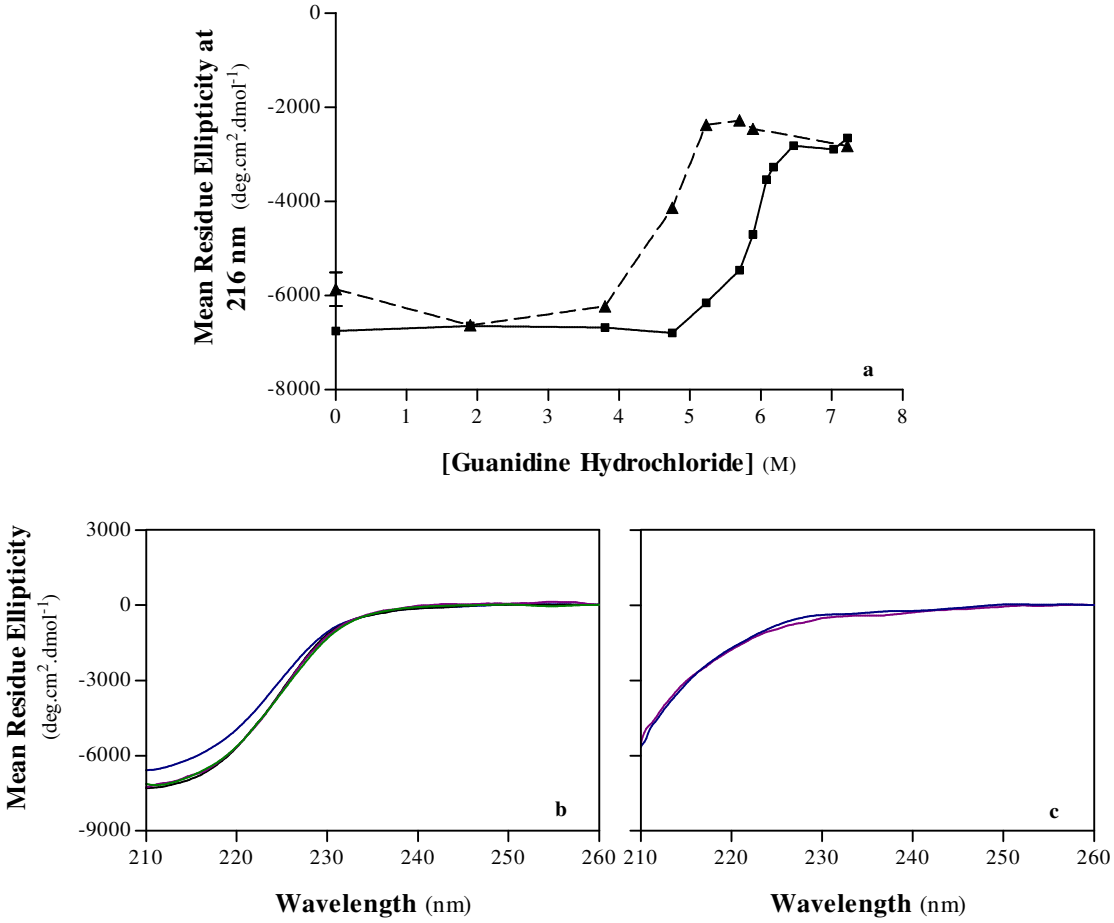

Figure 4.33 – **(a)** Dependence of the mean residue ellipticity of porcine pepsin A at 216 nm on the concentration of guanidine hydrochloride. Data were obtained from far-UV circular dichroic spectra, which were recorded after 1 h (squares; ___) and 3 days (triangles; _ _) of exposure of porcine pepsin A to increasing amounts of chaotrope at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot. **(b)** Far-UV circular dichroic spectra of porcine pepsin A recorded after incubation at 25ºC for 1 h in the absence (___) and in the presence of 1.90 M guanidine hydrochloride (___), and for 3 days in the absence (___) and in the presence of 1.90 M guanidine hydrochloride (___). **(c)** Far-UV circular dichroic spectra of porcine pepsin A collected after incubation at 25ºC in the presence of 7.22 M guanidine hydrochloride for 1 h (___) and 3 days (___).

The first conclusion following directly from the analysis of the Figure 4.33(a) is that the presence of guanidine hydrochloride at low concentrations might have hampered a drop in the contents of secondary structural elements, which was noted to occur upon 3 days under native-like conditions. In fact, spectra of porcine pepsin A generated after incubation in denaturant-free medium for 1 h, and in mixed solvent containing 1.90 M of charged denaturant for 1 h and 3 days overlapped. On the other hand, the average spectrum corresponding to porcine pepsin A left for 3 days in an aqueous solution of 10 mM sodium acetate at pH 3 registered subtle decay of secondary structure, in comparison with spectra generated under the same conditions at 1 h of incubation [see Figure 4.33(b)].

At the third day of incubation, a prominent loss of ordered structural units in the protein took place on going from 3.80 to 5.23 M guanidine hydrochloride, for absolute values of mean residue ellipticity of porcine pepsin A underwent a decrease at every wavelength in the range from 210 to 230 nm on spanning the

abovementioned interval of denaturant concentrations. The following inferences can be drawn from Figure 4.33(a) in regard to the time-dependence of gross disordering transition in porcine pepsin A mediated by guanidine hydrochloride: on prolonging the incubation time, the transition was onset at a lower denaturant concentration and occurred within a narrower range of denaturant concentrations. Consequently, the post-transition region began at an inferior concentration of guanidine hydrochloride, when the period of exposure to growing amounts of chaotrope lasted 3 days. However, it is worth highlighting that, regardless of the length of the incubation step, the end-point of guanidine hydrochloride - induced unfolding of porcine pepsin A remained similar in terms of backbone order. Indeed, Figure 4.33(c) shows superposition of spectra of porcine pepsin A in 7.22 M guanidine hydrochloride collected at both times of incubation. Therefore, longer incubation in highly concentrated solutions of denaturing salt did not allow reaching completeness of protein unfolding.

In a set of assays, the influence of acetonitrile on the response of the pepsin secondary structure to a few concentrations of guanidine hydrochloride was assessed. Experimental data regarding this issue are presented in the Figure 4.34. Acetonitrile concentrations selected for this study were 1.90 and 5.70 M, which corresponded to values, respectively, within the pre-transition region and at the lowest end of the transition region identified in the curve of the Figure 4.3(a).

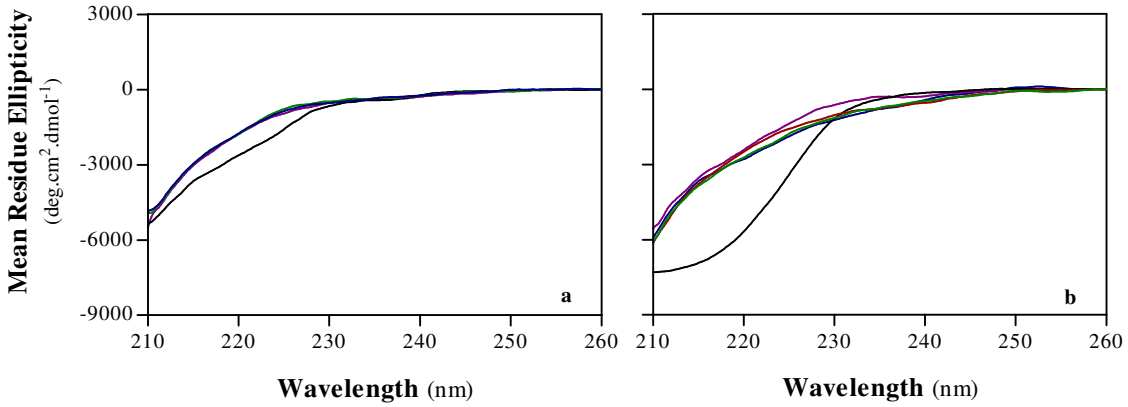

Figure 4.34 – Changes in the secondary structure of porcine pepsin A due to incubation in aqueous-organic mixtures containing guanidine hydrochloride at 25ºC for 1 h. The aqueous component of the solvent system was 10 mM sodium acetate at pH 3. **(a)** Far-UV circular dichroic spectra of porcine pepsin A generated after 1 h - incubation at 25ºC in: 6.07 M guanidine hydrochloride (___); 7.22 M guanidine hydrochloride (___); mixed medium containing 1.90 M acetonitrile and 6.07 M guanidine hydrochloride (___); and mixed medium containing 1.90 M acetonitrile and 6.76 M guanidine hydrochloride (___). The three latter spectra are superimposed. **(b)** Far-UV circular dichroic spectra of porcine pepsin A generated after 1 h - incubation at 25ºC in: free-denaturant aqueous medium (___); 6.18 M guanidine hydrochloride (___); mixed medium containing 5.70 M acetonitrile and 3.53 M guanidine hydrochloride (___); mixed medium containing 5.70 M acetonitrile and 4.03 M guanidine hydrochloride (___); and mixed medium containing 5.70 M acetonitrile and 4.54 M guanidine hydrochloride (___).

As can be seen from Figure 4.34(a), denatured conformations adopted by porcine pepsin A at highly concentrated solutions of guanidine hydrochloride in the absence and presence of 1.90 M acetonitrile were alike in terms of residual secondary structure. Nonetheless, protein disordering at 6.07 M of denaturing salt [a

concentration value within the transition region identified in the denaturation curve determined at 1 h of incubation, see Figure 4.33(a)] was intensified as a consequence of addition of 1.90 M acetonitrile to the incubation medium. This is claimed on the basis of larger values of mean residue ellipticity of porcine pepsin A previously incubated in an aqueous mixture of 1.90 M acetonitrile and 6.07 M guanidine hydrochloride *vis-à-vis* an identical mixture without organic solvent. In fact, on being simultaneously submitted to such amounts of organic solvent and chaotrope during 1 h, the pepsin secondary structure became comparable to that of the denatured state detected only after a similar period of incubation in aqueous solutions of guanidine hydrochloride at concentrations equal to and above 6.46 M [see Figure 4.34(a)].

Addition of 5.70 M acetonitrile to incubation media containing guanidine hydrochloride at pre-transitional concentrations [as judged from the denaturation curve determined at 1 h of incubation, see Figure 4.33(a)] caused remarkable disappearance of regular secondary structural elements. This is evidenced by spectra acquired for porcine pepsin A in non-aqueous solutions of guanidine hydrochloride at final concentrations equal to 3.53, 4.03 and 4.54 M for 1 h, which were very close to those collected for the protein under the effect of an aqueous solution of guanidine hydrochloride at 6.18 M during an equivalent period of time [see Figure 4.34(b)]. Overlapping of circular dichroic spectra corresponding to protein incubated in the aforementioned ternary mixtures suggests that acetonitrile at 5.70 M was capable of inducing the formation of a discrete partially unfolded conformational state, at least, in the 3.53 – 4.54 M interval of chaotrope concentrations. At first sight, the degree of disorder attained by porcine pepsin A in such intermediary state might not be expected. In response to aqueous mixtures of 5.70 M acetonitrile and 3.53 – 4.54 M guanidine hydrochloride, the mean residue ellipticity at 216 nm of native porcine pepsin A underwent, on average, a reduction of 48.45%. Bearing in mind that: (i) 1 h - incubation in 5.70 M acetonitrile caused only minor depletion of the contents of α-helices and β-sheets [see Figure 4.3(a)]; and (ii) spectra generated after exposure of porcine pepsin A to concentrations of guanidine hydrochloride equal to 3.80 and 4.75 M for 1 h were very similar to those collected for the protein under native-like conditions [see Figure 4.23(a)]; it is deemed that the impact of conjunct action of organic solvent and denaturing salt outweighed a summation of the impacts of each solvent condition isolated.

Spectroscopic data resultant from reappraisal of the dependence of secondary structure of porcine pepsin A on urea molarity at the third day of incubation are summarized in the Figure 4.35.

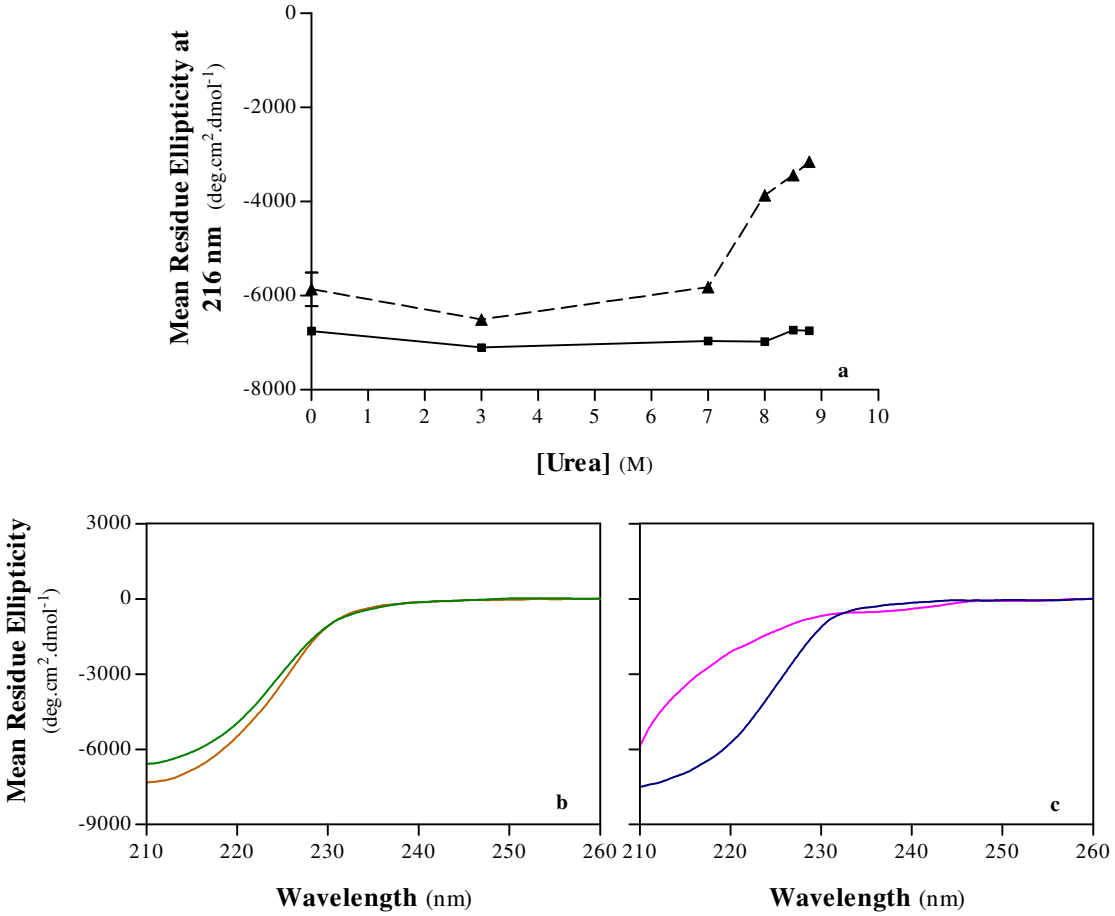

Figure 4.35 – **(a)** Influence of urea on the mean residue ellipticity of porcine pepsin A at 216 nm. Lines were merely used to connect the symbols. Data were obtained from far-UV circular dichroic spectra, which were recorded after 1 h (squares; ___) and 3 days (triangles; _ _) of exposure of porcine pepsin A to varying concentrations of the chaotrope at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. Vertical error bars symbolize standard errors of the mean. In case an error bar is not visible, it is smaller than the symbol in the plot. **(b)** Far-UV circular dichroic spectra of porcine pepsin A generated after incubation at 25ºC for 3 days in the absence (___) and in the presence of 3.00 M urea (___). **(c)** Far-UV circular dichroic spectra of porcine pepsin A collected after incubation at 25ºC for 1 h (___) and 3 days (___) in the presence of 8.79 M urea.

A glance at Figure 4.35(a, c) suggests that a longer period of incubation in aqueous solutions of urea favoured its disordering action on porcine pepsin A, even though only near-saturating concentrations of this neutral compound could bring about noteworthy conformational changes. In opposition to observations at 1 h of exposure to urea concentrations equal to and higher than 8.00 M, data collected at 3 days revealed an appreciable loss of backbone order. Nonetheless, the protein at the highest concentration of chaotrope tested, 8.79 M, still preserved 46.82% of the value of mean residue ellipticity at 216 nm typical for the natively folded state [see Figure 4.35(a, c)]. Thereafter, the final guanidine hydrochloride - denatured state of porcine pepsin A exhibited only slightly lower contents of secondary structural elements than that displayed by the protein after being under the effect of 8.79 M urea for 3 days.

Comparative analyses of spectra acquired in aqueous medium without and with urea at 3.00 M at 1 h and 3 days of previous incubation revealed resembling trends in the spectral behaviour of porcine pepsin A on changing solvent composition. This is, the polypeptide backbone remained more ordered in the presence

of 3.00 M of chaotrope than in aqueous medium lacking urea at both incubation times [see Figure 4.35(a, b)]. There was, however, a small decrease in the magnitude of the negative band in the circular dichroic spectrum collected in the amide region, on prolonging exposure to 3.00 M of chaotrope from 1 h to 3 days. In view of these results, it is conceivable that the non-native secondary structure, which was induced by such low molarity of urea and observed after 1 h of exposure, endowed the protein with greater resistance against slight unfolding detected in reference conditions (*i. e.*, slight unfolding earlier reported to occur in the protein incubated in aqueous medium during 3 days).

E.1.2. *Tertiary Structure*

Herein, responses of the native tertiary structure of porcine pepsin A to exposure to guanidine hydrochloride (see Figure 4.36) and urea (see Figure 4.37) at varying molarities for 3 days, as followed by near-UV circular dichroism, will be briefly described, in comparison with analogous data acquired after a shorter period of incubation (1 h) in matching solvent conditions.

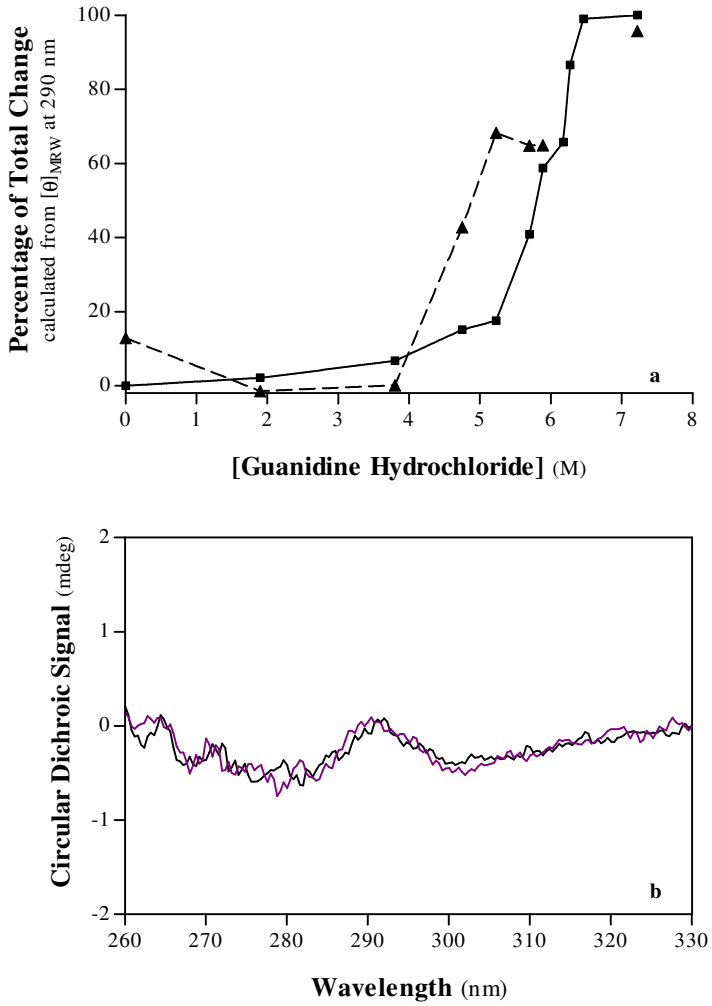

Figure 4.36 – **(a)** Alterations in the mean residue ellipticity of porcine pepsin A at 290 nm induced by guanidine hydrochloride expressed in terms of percentage of the total change occurring between 0 and 7.22 M of denaturant. Data were obtained from near-UV circular dichroic spectra, which were recorded after 1 h (squares; ___) and 3 days (triangles; _ _) of exposure of porcine pepsin A to increasing concentrations of the chaotrope at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. **(b)** Raw near-UV circular dichroic spectra of porcine pepsin A collected after incubation at 25ºC in 7.22 M guanidine hydrochloride for 1 h (___) and 3 days (___).

It was remarkable to observe that, on the contrary to data concerning 1 h - incubation, exposure of porcine pepsin A to 1.90 and 3.80 M guanidine hydrochloride (chaotrope concentrations lower than those required to onset a disordering transition) for 3 days gave rise to enhanced order in the vicinity of tryptophan side chains, as compared to the structural microenvironment of these aromatic residues in the protein under native-like conditions for the same period of time [see Figure 4.36(a)]. Moreover, a longer period of exposure to such low concentrations of guanidine hydrochloride brought about small increments in the circular dichroic absorbance at 290 nm observed at 1 h, with larger differences at 3.80 M of chaotrope; whereas a comparative analysis of spectra of porcine pepsin A in a denaturant-free medium evidenced a structural

reorganization between 1 h and 3 days, which led to the tryptophan side chains being less rigidly held in the protein three-dimensional structure. Taken as a whole, these results suggest that the capability for low molarities of guanidine hydrochloride to promote local order or disorder varied along the course of incubation. Initially, addition of 1.90 and 3.80 M guanidine hydrochloride provoked modest unpacking of indole side chains as evidenced by spectra recorded after 1 h of incubation. However, local structural rearrangements must have occurred between the first hour and the third day of exposure to low concentrations of guanidine hydrochloride (especially, at 3.80 M) that caused a depletion in the mobility of tryptophan side chains. Constrained environments of these aromatic residues in the presence of 1.90 and 3.80 M of denaturing salt after 3 days of incubation are predicted to be similar to native ones.

As proved by Figure 4.36(a), structural perturbation induced by 4.75 M guanidine hydrochloride was substantially higher at the third day than at the first hour of incubation. Thereafter, a reduction in the denaturant concentration required for triggering severe unfolding followed as a consequence of extending the period of exposure to solvent conditions.

The hypothesis earlier raised for the occurrence of an intermediary conformational state in the unfolding pathway of porcine pepsin A assisted by guanidine hydrochloride was confirmed by measurements of circular dichroic absorbance in the near-UV region after 3 days of incubation in mildly concentrated solutions of denaturant. Such conformational state was isolatable in the concentration interval of 5.23 – 5.89 M, and corresponded to 65.99% of total change in the local conformation nearby tryptophan residues [see Figure 4.36(a)]. In regard to the final guanidine hydrochloride - denatured states, the degree of disorder in the tertiary arrangement is deemed to be comparable in unfolding profiles measured after 1 h and 3 days of incubation. Evidence in favour of this surmise comes from close proximity of the overall shapes of corresponding spectra [see Figure 4.36(b)].

At the third day of incubation, enhanced denaturing potential of urea against porcine pepsin A was manifested at the level of, not only secondary structure (as previously described in this section), but also tertiary structure as reflected by Figure 4.37.

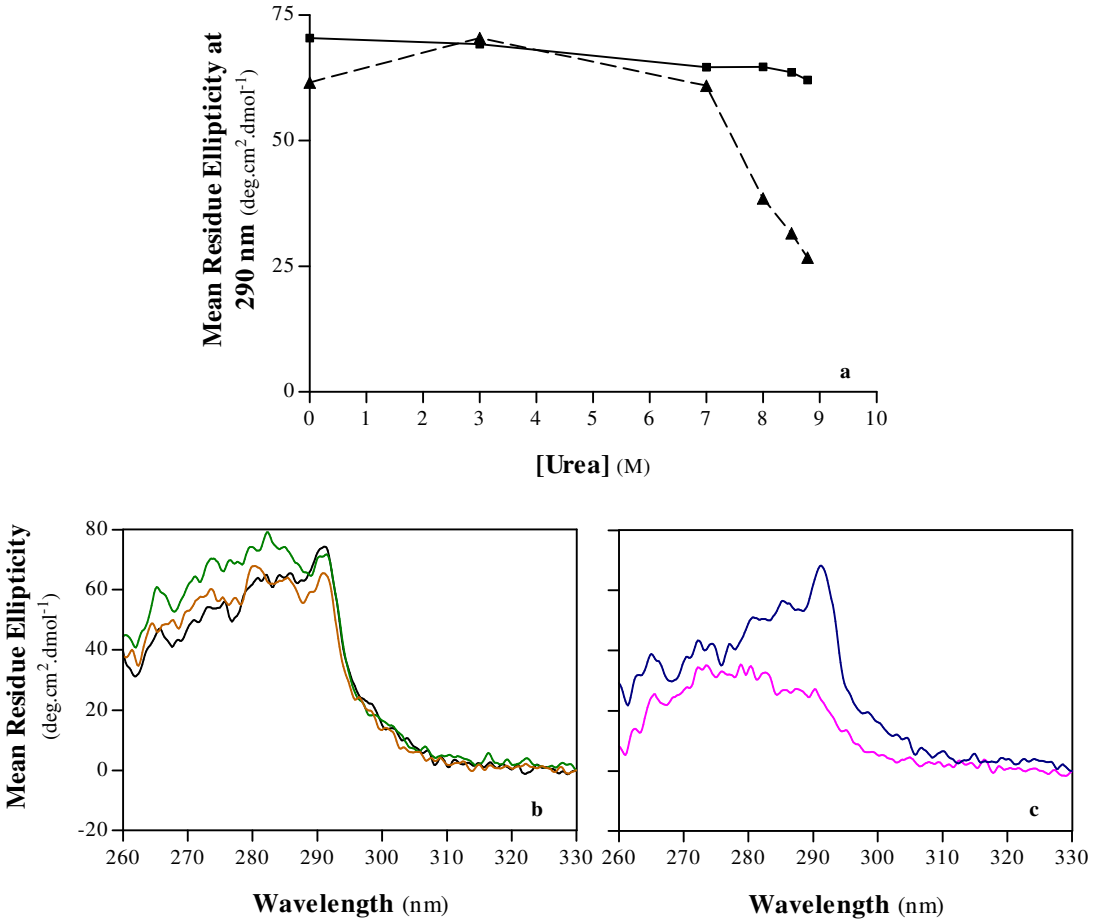

Figure 4.37 – **(a)** Dependence of the mean residue ellipticity of porcine pepsin A at 290 nm on urea concentration. Lines were merely used to connect the symbols. Data were obtained from near-UV circular dichroic spectra, which were recorded after 1 h (squares; ___) and 3 days (triangles; _ _) of exposure of porcine pepsin A to varying concentrations of the chaotrope at 25ºC. An aqueous solution of 10 mM sodium acetate at pH 3 was used as reference medium and as solvent for preparation of denaturant mixtures. **(b)** Near-UV circular dichroic spectra of porcine pepsin A generated after incubation at 25ºC for 1 h in aqueous medium (___), and for 3 days in aqueous medium (___) and in the presence of 3.00 M urea (___). **(c)** Near-UV circular dichroic spectra of porcine pepsin A generated after incubation at 25ºC for 1 h (___) and 3 days (___) in the presence of 8.79 M urea.

Protein samples incubated in the presence of urea concentrations equal to and above 8.00 M for 3 days before measurement gave rise to values of mean residue ellipticity in the aromatic region significantly lower than protein samples under the effect of identical or native-like solvent conditions during 1 h. In other words, near-saturating concentrations of urea could induce large-scale alterations in the native tertiary arrangement in the period between 1 h and 3 days, whereas similar amounts of chaotrope appeared to be rather ineffective in that sense from 0 to 1 h of incubation [see Figure 4.37(a, c)]. Despite severe perturbation in tertiary structure, there is evidence for protein denatured conformations induced by exposure to high molarities of urea for 3 days to retain aromatic residues considerably constrained in hydrophobic cores [see Figure 4.37(c)], which is in clear contrast with the trivial tertiary structure detected in the final guanidine hydrochloride - state of porcine pepsin A.

Once more, the response of porcine pepsin A to low urea concentrations apparently contradicted the

traditional conception of this compound as a disordering agent. On generating a spectrum of the protein upon exposure to 3.00 M urea for 3 days, one could detect aromatic bands with larger magnitude in relation to bands in a spectrum collected for a denaturant-free pepsin sample after the same time of incubation. These observations are indicative of greater degree of order in the overall tertiary structure of porcine pepsin A in the presence of urea at the third day of incubation [see Figure 4.37(b)]. Notwithstanding the level of asymmetry in the microenvironment of tryptophan residues appears to have remained native-like during the 3 days of incubation in 3.00 M urea [*i. e.*, values of spectroscopic signal at 290 nm collected for the protein after 1 h in aqueous medium with and without 3.00 M of denaturant and after 3 days in aqueous medium with 3.00 M of denaturant were similar, see Figure 4.37(a)]; a part of the protein molecule, most certainly, suffered conformational rearrangements, resulting in stronger tertiary interactions in the neighbourhood of some aromatic residues which, in consequence, might be occupying more fixed positions. This proposal is consistent with the fact that more intense aromatic bands in the spectrum corresponding to the protein incubated in 3.00 M urea for 3 days, as compared with the typical native spectrum, were observed only below 288.6 nm [see Figure 4.37(b)].

E.1.3. *Interpretative Remarks*

E.1.3.1. *Prolonged Incubation in Aqueous Solutions of Chaotropes*

Notwithstanding extension of the period of exposure of natively folded porcine pepsin A to both chaotropic agents at low molarities revealed a few apparent similarities in their general mechanisms of action, the extent of unfolding achieved by the protein in mildly or highly concentrated solutions of guanidine hydrochloride still far outweighed that attained in equimolar solutions of urea (see Figures 4.33, 4.35, 4.36 and 4.37). Besides, data obtained for the influence of both denaturants on protein conformation, previously described in the ongoing section, have evidenced their double-edged actions. On one hand, spectroscopic measurements of samples of porcine pepsin A at the third day of incubation under the selected experimental conditions allowed identifying native-like forms with greater degrees of structural organization at low concentrations of both the electrolyte and the non-electrolyte as compared to that displayed by the protein in denaturant-free aqueous medium. On the other hand, an increase in harshness of the medium revealed clear disordering effects by both denaturants, which involved conformational changes in native porcine pepsin A more drastic in relation to those associated with the non-conventional effects observed at low molarities of both chaotropes. On opting for a longer incubation step, the ability for promoting structural disarray was emphasized, in the sense that: (i) lower concentrations of guanidine hydrochloride were required to detect gross unfolding; and (ii) more pronounced disordering conformational changes were observed in urea-rich media. Divergent co-solvent effects on porcine pepsin A shall be looked at in separate.

E.1.3.1.1. *Non-Conventional Effects of Chaotropes: Inhibition of Unfolding and Enhancement of Packing*

At the third day of incubation, differences encountered in the behaviour of porcine pepsin A in the absence and presence of denaturants at low molarities are at odds with the established notion of guanidine hydrochloride and urea as chaotropic agents. In summary, lowly concentrated solutions of the charged and the uncharged denaturant apparently endowed the protein with conformational resistance to minor unfolding, which was observed at the third day in denaturant-free aqueous medium. This phenomenon entailed: (i) total or partial inhibition of decay of secondary structure [see Figures 4.33(a, b) and 4.35(a, b)], and (ii) gain [see Figure 4.37(b)] or regain [see Figure 4.36(a)] in tertiary structural organization as judged from increases in order in the vicinity of aromatic side chains.

One plausible explanation for the observations just described relates to the capability of guanidine hydrochloride and urea to act as cross-linking agents, *i. e.*, to connect different parts of the protein by establishing multiple and variable-length hydrogen bonds and van der Waals interactions with main chain and side chain groups of proteins (Dunbar, *et al.*, 1997; Kumar, *et al.*, 2004; Pike & Acharya, 1994), when used at pre-unfolding concentrations. In so doing, these chaotropes promote protein stiffening; in other words, they afford an increase in barriers to motions and, thus, impose a depletion in conformational flexibility (Kumar, *et al.*, 2004; Pace, *et al.*, 2005).

The ionic nature of guanidine hydrochloride often provides a basis for discrimination between denaturing abilities of such compound and urea. Indeed, the effect of guanidine hydrochloride on protein conformation and stability has been deconvoluted into denaturant and electrolyte effects (Smith & Scholtz, 1996). On adsorbing on the protein surface due to its preferential interaction with hydrogen-bonding groups, it is conceivable that the guanidinium ion can mask charged side chains and, hence, weaken or totally eliminate optimized electrostatic interactions (Del Vecchio, *et al.*, 2002; Monera, *et al.*, 1994; Schimmele & Plückthun, 2005). The effect of screening electrostatic interactions on protein conformation and stability should depend on whether they are repulsive or attractive, and on their relative importance to the protein (Monera, *et al.*, 1994). Bearing this in mind, an electrostatic effect due to interaction of guanidinium ions (and chloride ions) with charged groups has been deemed a source of protein stabilization at low denaturant concentrations, whenever a situation is favourable for charge screening; even though an entropic effect due to the intramolecular cross-linking action of the cation invariably operates (Bhuyan, 2002; Kumar, *et al.*, 2004; Shukla, *et al.*, 2005).

Salt effects might have been involved in the non-linear response of pepsin tertiary structure to low molarities of guanidine hydrochloride (particularly, to 3.80 M; see Figure 4.36) along the course of incubation. From 0 to 1 h, the charged denaturant caused a minor disarray in the tertiary structure. Subsequently, porcine pepsin A underwent a structural rearrangement, so that the local structural environments of aromatic residues regained order and became practically native-like at the third day of incubation. The protein conformational behaviour between 1 h and 3 days of exposure to low concentrations

of denaturant could be explained as follows: initial loosening of the protein might have caused changes in the relative positions and / or in charges of a few acidic residues nearby aromatic residues, which propitiated repulsive electrostatic interactions. Guanidinium ions could have suppressed repulsion by masking negatively charged side chains, thereby re-establishing order in the microenvironment of aromatic side chains. Experimental support for this speculative interpretation remains to be seen.

Particular attention should be drawn to the behaviour of porcine pepsin A in the presence of 1.90 M guanidine hydrochloride, because neither secondary nor tertiary structures at the third day of incubation exhibited major differences in comparison with corresponding native arrangements. In other words, changes induced by guanidine hydrochloride within a period of 3 days appear to have endowed the protein with greater resistance against unfolding at a global level. This phenomenon, however, can only be referred to as *apparent stabilization* for there is no available evidence suggestive of an effective increase in thermodynamic conformational stability.

E.1.3.1.2. *Conventional Effects of Chaotropes: Induction of Unfolding*

E.1.3.1.2.1. *Guanidine Hydrochloride*

Denaturation curves determined at the third day of incubation conveyed that, as more severe conditions were approached, the unfolding effect of guanidine hydrochloride overrode its own apparent stabilizing effect. Differences between unfolding profiles obtained after different periods of exposure to the denaturant, namely, in terms of shape and range of transitional concentrations, confirmed that a 1 h - incubation was not enough for attaining equilibrium at all molarities of guanidine hydrochloride [see Figures 4.33(a) and 4.36(a)].

Apart from the length of the incubation step, other variables should influence profiles of guanidine hydrochloride - induced unfolding of porcine pepsin A, such as pH. In a study conducted by Yoshimasu and co-workers (2004), wild-type pepsin at pH 5.3 was submitted to increasing amounts of guanidine hydrochloride for 9 h and 25ºC, and resultant conformational changes were monitored by probes of conformation and activity. Denaturation curves were found to be sigmoidal. Transitional phases were steep and narrow, for they took place in the 1.75 – 2.70 M interval of concentrations of guanidine hydrochloride (Yoshimasu, *et al.*, 2004). Campos and Sancho (2003) managed to identify an intermediary conformational state of pepsin in the pH range from 4 to 6.5 from pH titration curves measured after a 5 h - incubation at 25ºC. In view of this, the experiments performed by Yoshimasu and collaborators (2004) targeted pepsin under non-native conditions. Campos and Sancho (2003) further stated that the transition between the natively folded state and the intermediate dominant at mildly acidic pH entailed exposure of tryptophan residues and an increase in secondary structure contents. Comparison of data regarding guanidine hydrochloride - unfolding obtained for the natively folded [see Figures 4.33(a) and 4.36(a)] and the pH-induced intermediary (Yoshimasu, *et al.*, 2004) conformational states suggests that the non-native structure acquired by the latter state decreased the threshold for unfolding, since gross disordering of pepsin at pH 3

was detected only at chaotrope concentrations higher than 3.80 M (as judged from data obtained at the third day of incubation).

On prolonging the period of exposure to denaturing conditions, it was possible to ascertain that the unfolding pathway of porcine pepsin A induced by the charged denaturant did not conform to a two-state model. A stable, partially unfolded conformational state could be isolated in the range of denaturant concentrations from 5.23 to 5.89 M. Earlier studies on guanidine hydrochloride - provoked denaturation of pepsin were not in agreement in regard to the degree of cooperativity in this process. Some authors found the conformational transition to be cooperative (Ahmad & McPhie, 1978; Yoshimasu, *et al.*, 2004), whilst others alluded to a stepwise unfolding reaction (Pain, *et al.*, 1985b). Either way, the author of this study is unaware of a description of an intermediate state stabilized by guanidine hydrochloride in the unfolding pathway of porcine pepsin A available at the open literature. From structural data obtained in this research, one can conclude that it retained substantial contents of secondary structural elements [which are similar to those observed in the final denatured state; see Figure 4.33(a)] and tertiary packing [in opposition to the final denatured state; see Figure 4.36(a)].

Multiphasic conformational transitions might suggest that different regions in the protein structure have different conformational stabilities. Indeed, from calorimetric studies on pepsin at near-neutral pH values, Privalov and collaborators (1981) computed different thermodynamic conformational stabilities for each pepsin domain, with the N-terminal domain being less stable than the C-terminal domain. Nevertheless, in the case of the guanidine hydrochloride - mediated unfolding of porcine pepsin A, there is another plausible explanation for the accumulation of an intermediate, which again calls into play electrostatic effects of the chaotrope. Porcine pepsin A is rich in aspartic and glutamic residues. About 20 out of 43 are buried or partially buried in the protein three-dimensional structure. It is likely that repulsive interactions increase on unfolding, due to higher exposure of acidic residues. Thereafter, stabilization of the partially unfolded state of porcine pepsin A by guanidine hydrochloride could have originated from mitigation of repulsive forces between charged groups closely positioned by the denaturant. This proposal awaits experimental validation. Nonetheless, this is not the first study assigning accumulation of an intermediate in a pathway of protein unfolding to screening of charges by guanidine hydrochloride (Srimathi, *et al.*, 2003).

Even though the disordering action of guanidine hydrochloride on the protein seems to have been intensified by an increase in the period of exposure to the chaotrope, final denatured states identified in unfolding profiles determined at 1 h and 3 days of incubation were structurally equivalent. The end-point of guanidine hydrochloride - induced unfolding of porcine pepsin A was similar to the partially unfolded state in terms of secondary structure [see Figure 4.33(a)]. Nevertheless, the tertiary arrangement was almost entirely disrupted [see Figure 4.36(b)]. This was taken to mean that it is possible for regular elements of secondary structure to exist even in the absence of well-defined tertiary structure. Data described herein are, however, agnostic about whether ordered units in the unfolded state are of native-like geometry.

Detection of non-random structure in the final guanidine hydrochloride - denatured state is

consistent with new unfolded-state models. The traditional conception of denatured states in highly concentrated solutions of classic chemical denaturants as fully unstructured, randomly fluctuating polymers is being re-formulated. There is mounting evidence pointing to chaotrope-unfolded states as being incompletely disordered, and containing native and non-native residual secondary structure (Bhavesh, *et al.*, 2003; Bhavesh, *et al.*, 2004; Chatterjee, *et al.*, 2005), hydrophobic clustering (Chatterjee, *et al.*, 2005; Saab-Rincon, *et al.*, 1996; Shimizu & Chan, 2002) and elements of native-like topology (Shortle & Ackerman, 2001).

E.1.3.1.2.2. *Urea*

After a 3 days - incubation, disordering of secondary and tertiary arrangements revealed to be abrupt and nearly coincidental upon submission of porcine pepsin A to urea concentrations above 7.00 M. In other words, instead of resistance of backbone order in urea-rich aqueous solutions observed at 1 h, one could detect its substantial decay approximately 3 days after (see Figure 4.35). And, damages in the pepsin tertiary structure were severely aggravated on extending the period of incubation in highly concentrated solutions of denaturant (see Figure 4.37). On balance, onset of gross structural perturbation of natively folded porcine pepsin A by urea required, not only near-saturating concentrations of chaotrope, but also a long phase of incubation in the denaturing medium. This was taken to mean that the rate of unfolding of native porcine pepsin A assisted by urea was very slow.

Greater conformational changes in porcine pepsin A after longer incubation in urea-rich media could follow from intensification of the indirect mechanism of action of the chaotrope and its higher accessibility to the protein surface, facilitating solvation of non-polar areas and backbone and, hence, accelerating unfolding. In consonance with what has been mentioned, several factors might be responsible for differences in the denaturing potential of guanidine hydrochloride and urea. However, their mechanisms of action have been suggested to share many similarities. Accordingly, it is expected that destabilization of secondary and tertiary structures of porcine pepsin A by urea was as well driven by disruption of non-covalent intramolecular interactions within the protein, and solvation of main chain and side chain groups.

It is interesting to realize that large-scale denaturation, embracing both secondary and tertiary arrangements, observed at the third day of incubation was preceded by a modest depletion in packing of aromatic residues (whose majority are buried or partially buried in protein hydrophobic cores) and in solvent shielding of, specifically, tryptophan residues as observed at 1 h [see Figures 4.25 and 4.26(b, c)]. Slight debilitation of tertiary structure prior to gross unfolding had already been noted in the response of porcine pepsin A to guanidine hydrochloride. It is conceivable that minor weakening of solvent-mediated hydrophobic interactions, owing to urea-induced disordering of water molecules in solvation structures, caused a subtle loosening of the protein and, thus, a decrease in the threshold for major destruction of ordered arrangements of non-polar side chains and backbone by promoting access of solvent to the periphery of the hydrophobic cores. This hypothetical scenario could be consistent with a model for chemical denaturation of proteins by urea proposed by Bennion and Daggett (2003).

E.1.3.2. *Incubation in Non-Aqueous Solutions of Guanidine Hydrochloride*

The final guanidine hydrochloride - denatured state of porcine pepsin A was not affected by the presence of 1.90 M acetonitrile [see Figure 4.34(a)]. On the other hand, after a 1 h - incubation in a mixture of 1.90 M acetonitrile and 6.07 M guanidine hydrochloride [which is a concentration value within the transition phase of the curve corresponding to unfolding of porcine pepsin A induced by guanidine hydrochloride depicted in the Figure 4.23(a)], the residual secondary structure of porcine pepsin A is comparable to that exhibited by the unfolded state [see Figure 4.34(a)]. It should be stressed that, from denaturation profiles obtained after exposure of the protein to aqueous solutions of chaotrope for 1 h, the guanidine hydrochloride - unfolded state could only be detected at chaotrope concentrations higher than 6.46 M. It can be concluded that acetonitrile and guanidine hydrochloride had a cumulative effect on the native protein.

An outstanding combined effect could be observed when 5.70 M of organic solvent was added to protein-solvent systems containing denaturing salt at concentrations in the range from 3.53 to 4.54 M. Apparently, the conjunct action of the two denaturants far surpassed the sum of their isolated actions [see Figure 4.34(b)]. Indeed, submission of porcine pepsin A solely to guanidine hydrochloride concentrations in the 3.53 – 4.54 M interval for 1 h did not induce changes in the secondary structure detectable by spectroscopic probes, and exposure to 5.70 M acetonitrile for an equal period of time did only provoke a minute reduction in β-sheets and α-helices in porcine pepsin A. However, porcine pepsin A was noted to undergo a visible reduction in its Stokes radius in the presence of this amount of organic solvent, which was taken to be caused by dehydration. A weaker solvation shell should allow better access of the poorly hydrated guanidinium cation to the protein surface and, hence, fasten unfolding.

CHAPTER V

GENERAL DISCUSSION

Pivotal roles in a wide range of pathological conditions which are becoming major causes of death in both developed and developing worlds have been attributed to several aspartic proteinases. Consequently, this group of enzymes is being target of much interest. Improvement and / or discovery of clinical solutions are fuelling efforts on broadening knowledge about aspartic proteinases. In particular, the development of enzyme inhibitors as therapeutic agents (for reviews, see the following references: Cooper, 2002; Dash, *et al.*, 2003) has impelled resolution of three-dimensional structures of enzymes and / or enzyme-inhibitor complexes (Bailey & Cooper, 1994; Foundling, *et al.*, 1987; Sielecki, *et al.*, 1989), elucidation of the catalytic mechanism (Beveridge & Heywood, 1993; Das, *et al.*, 2006; Kumar, *et al.*, 2005) and establishment of structure-function relationships using protein engineering and mutagenesis approaches (Bryksa, *et al.*, 2003; Griffiths, *et al.*, 1994; Lin, *et al.*, 1993; Scarborough & Dunn, 1994; Tanaka & Yada, 2001). On the other hand, folding / unfolding processes and conformational stability are still poorly explored within the group of aspartic proteinases. Incentives for more intensive research in these fields are, for example, perspectives for engendering strategies for protein modulation in terms of conformation and biophysical properties with possible consequences in function, or for understanding implications of molecular evolution in folding reactions and conformational stability.

From the limited set of published studies regarding folding / unfolding processes and conformational stability of aspartic proteinases, many focus on HIV-1 proteinase (Bhavesh, *et al.*, 2003; Cheng, *et al.*, 1990; Grant, *et al.*, 1992; Szeltner & Polgár, 1996), mammalian cathepsin D (Lah, *et al.*, 1983; Lah, *et al.*, 1984; Pain, *et al.*, 1985a; Turk, *et al.*, 1981), cardosin A (Oliveira, 2007; Pina, *et al.*, 2003; Shnyrova, *et al.*, 2006) and some fungal enzymes (Beldarraín, *et al.*, 2000; Tello-Solís & Hernandez-Arana, 1995). On account of being treated as an archetypal enzyme in the group of aspartic proteinases, it is not surprising that unfolding pathways and conformational stability of pepsin (*vide*, for example, Ahmad & McPhie, 1978; Campos & Sancho, 2003; Dee, *et al.*, 2006; McPhie, 1989; Privalov, *et al.*, 1981) and its zymogenic form (Edelhoch, *et al.*, 1965; Frattali, *et al.*, 1965; McPhie, 1982; McPhie & Shrager, 1992; Privalov, *et al.*, 1981; Makarov, *et al.*, 1995) have as well been investigated. Many studies, however, targeted inhibited or free, non-native

pepsin. By contrast, herein, the object under investigation was free, natively folded pepsin, in an attempt to gain more straightforward insights into the mechanisms responsible for the maintenance of its dominant, ordered arrangement under physiological conditions.

It is worth emphasizing that the concept of native state of pepsin refers to the conformational state sampled by the protein in the stomach lumen (or comparable experimental settings), where it is predominantly located and performs its physiological function. Clarification of this concept is imposed by a recent proposal for adoption of an additional, albeit transient, well-folded state by mature protein *in vivo*. After its secretion from chief cells, pepsinogen undergoes activation in the deepest regions of gastric glands (*i. e.* at pH equal to or lower than 3) and, as a result, natively folded and biologically active pepsin is generated (Campos & Sancho, 2003; Gritti, *et al.*, 2000). On its way to the stomach lumen through a gastric gland and its corresponding crypt, pepsin crosses a pH gradient, whereby a pH around 4.6 is attained at the outlet of the gastric crypt (Schreiber, *et al.*, 2000). On reaching regions of pH higher than 4, pepsin has been suggested to assume an intermediary state. Even though such conformational state is argued to have native-like characteristics; it displays higher contents of secondary structural elements and a lower degree of tryptophan burial as compared to the natively folded state. Moreover, its enzymatic activity is impaired. Adoption of such intermediary state has been proposed to be essential for transport of pepsin to the location where the enzyme plays its biological role. The environmental pH is lowered in regions proximal to the stomach lumen, where the enzyme is reconverted into its natively ordered and biologically active state (Campos & Sancho, 2003).

The abovementioned intermediary state was demonstrated to be isolatable through *in vitro*, equilibrium experiments in the 4 – 6.5 pH interval (Campos & Sancho, 2003). And, in the section 2.E. '*Induction of Denaturation under Non-Standard Experimental Conditions*' of chapter IV, it is concluded that such intermediate is less stable than the natively folded state. Several studies on pepsin overlook the occurrence of the well-folded intermediary species, and the enzyme is often deemed to assume its native state at mildly acidic pH (Dee, *et al.*, 2006; Kamatari, *et al.*, 2003; Konno, *et al.*, 2000; Yoshimasu, *et al.*, 2004). In order to withdraw valid conclusions from investigations on pepsin, it is advisable to unambiguously define whether reference experimental conditions (namely, environmental pH and length of incubation periods) propitiate acquirement of the unique, stable, natively ordered and biologically active state or an alternative, metastable state by the protein.

This chapter includes an overview of general features of denaturation of natively folded porcine pepsin A induced through direct modification of physical and chemical properties and / or change in composition of the solvent system by addition of chemical compounds. Perturbation of the fine balance of non-covalent interactions which sustains the natively folded conformational state of a protein is helpful in sketching a picture, not only on its unfolding pathway, but also on its energetics. In view of this, mergence of data on alterations in several specific properties of the protein in diverse environmental setups is hoped to provide a framework for building proposals regarding the conformational stability of native porcine pepsin A. Whenever it is considered feasible and useful, conclusions and hypotheses deriving from the study currently described will be reconciled and compared with information about this and other proteins available

at open literature.

1. *FROM DENATURATION TO CONFORMATIONAL STABILITY OF PORCINE PEPSIN A*

Bulk data obtained throughout this research, which were described and commented in the previous chapter, concern chemical denaturation of natively folded porcine pepsin A (at pH 3 and 25ºC). An organic solvent – acetonitrile, two classic chaotropes – guanidine hydrochloride and urea, and organic solvent-chaotrope mixtures were used as perturbants. Moreover, a few additional insights were furnished into the response of the protein to high temperature and a raise in pH. Variations in structural, hydrodynamic, biophysical and functional properties induced by the denaturing factors selected were examined. Results from this research hint at a diversity of denaturation scenarios and non-native forms.

A. *Large-Scale Denaturation*

A.1. *Chemical Compounds*

Mechanisms of action of organic solvents and chaotropes wait for full elucidation. Still, the ability of organic solvents and chaotropes to denature proteins has been mostly approached from two general viewpoints: (i) interception of water structure with consequences in the integrity of solvation structures; and (ii) direct interaction with the protein surface. And, dissimilarities in their effects on a protein are intuitively assigned to their chemical and physical properties. Guanidine hydrochloride is an electrolyte, whereas acetonitrile and urea are non-electrolytes. Acetonitrile is an amphiphilic solvent because it contains organic and polar functional groups (Dawson & Wallen, 2002). Guanidinium ion and urea are entirely polar, but they are able to interact with polar and non-polar moieties (Vanzi, *et al.*, 1998). The organic solvent is a non-hydrogen bond donor (Kang, *et al.*, 1994a; Kang, *et al.*, 1994b; Maroncelli, 1991; Olofsson, *et al.*, 2005) and its proton acceptor ability is weak (Ababneh, *et al.*, 2003; Venables & Schmuttenmaer, 1998). On the other hand, the hydrogen-bonding capacity of urea is higher than that of water (Pace, *et al.*, 2005; Soper, *et al.*, 2003), whilst the guanidinium ion can only form hydrogen bonds in its molecular plane (Dempsey, *et al.*, 2005).

Before launching into a comparative analysis of unfolding reactions promoted by each denaturant, it is worth recalling an important procedural detail: the protein was dissolved in 10 mM sodium acetate at pH 3 prior to incubation in denaturant mixtures. In other words, solvation structures rich in water were formed around the biomacromolecule in the initial step of protein dissolution in the aqueous medium. And, the

degree to which the hydration network was retained upon dilution in denaturant mixtures should depend on the affinity of each denaturing compound to water.

Unfolding profiles constructed from structural properties measured after a 1 h - incubation of porcine pepsin A in aqueous mixtures of each of the three denaturants were found to be different. While urea at near-saturating concentrations induced modest loosening of the tertiary structure [see Figures 4.25 and 4.26(b, c)], guanidine hydrochloride and acetonitrile promoted extensive disorder of backbone arrangement and loss of tertiary packing and interactions [see Figures 4.3, 4.6, 4.10, 4.23, 4.24, 4.26(a, c)].

Urea appears to be a good competitor for water-water hydrogen bonds. Each denaturant molecule has been estimated to establish, on average, 5.7 hydrogen bonds in urea-water mixtures at a molar ratio (urea:water) of 1:4. Furthermore, it has been reported to easily incorporate into the water network. On one hand, these two features are compatible with an effect of the non-electrolyte on the three-dimensional arrangement of water molecules (Soper, *et al.*, 2003). Disruption of water structure in the protein solvation sphere subsequent to addition of urea could lead to weakening of solvent-mediated hydrophobic interactions. On the other hand, considering the efficiency of urea molecules in substituting for water molecules in the hydrogen-bonded network of water, one could envisage a scenario where denaturant molecules would partially fulfil the role of water molecules in solvation structures of porcine pepsin A, thereby lowering the unfolding rate to a certain extent and allowing preservation of considerable structural organization.

A proposal for an important role of solvation structures in upholding the native conformational state of porcine pepsin A is withstood by a coincidence between a reduction in the protein hydrodynamic radius and the onset of major unfolding in the presence of acetonitrile (see section 1.B. '*Hydrodynamic Behaviour*' of the chapter IV). There is evidence for alterations in the arrangement of hydrogen-bonded molecules in pure water induced by this organic solvent (Bertie & Lan, 1997; Jerie, *et al.*, 2005; Roberston & Sugamori, 1972 as quoted by Jamroz, *et al.*, 1993 and Kovacs & Laaksonen, 1991; Venables & Schmuttenmaer, 1998). Acetonitrile has inclusively been demonstrated to be capable of displacing essential protein-bound water molecules (Yang, *et al.*, 2004). Yet, its power for solubilizing protein polar groups is, in general, weak, as judged by positive values of Gibbs free energy change associated with most cases of transfer of polar amino acids from water to aqueous mixtures of acetonitrile at 25ºC (Gekko, *et al.*, 1998). Besides, taking into account the low potential of acetonitrile for establishing hydrogen bonds (Ababneh, *et al.*, 2003), molecules of this organic solvent are expected not to succeed in replacing water molecules in the solvation shell of porcine pepsin A as effectively as urea. In view of this, mixed solvation shells including acetonitrile should be less strongly held by the protein, and this should contribute to a lower magnitude of the hydrophobic effect.

Curves of acetonitrile - and guanidine hydrochloride - denaturation determined after submission of natively folded porcine pepsin A to solvent conditions for 1 h indicate that extensive unfolding was onset at close concentrations of organic solvent and chaotrope. Interestingly, both denaturants are less competent to engage hydrogen bonds with water as compared to urea. Each guanidinium ion was found to form up to three undistorted hydrogen bonds with water molecules (Mason, *et al.*, 2003). According to Kovacs and Laaksonen (1991), two weak hydrogen bonds are established between one water molecule and two acetonitrile

molecules at low water contents; even though water is prone to self-association over a wide concentration range. It is, thus, tempting to assume that one of the causes for greater potential for denaturing proteins by guanidinium cation in comparison with urea is related to a low capability to efficiently replace water molecules in the solvation shell, just like it was suggested for acetonitrile.

On comparing studies on solubility and preferential interaction in denaturant mixtures, guanidine hydrochloride appears to be closer to the ideal solvent for proteins. These studies point to higher solubility of non-polar side chains and, in general, diminished solubility of polar groups in acetonitrile-water mixtures in relation to water (Gekko, *et al.*, 1998); whereas guanidinium ion and urea enhance solubility of most side and main chain groups compared to water (Nozaki & Tanford, 1963; Nozaki & Tanford, 1970; Qu, *et al.*, 1998). Protein solubility in aqueous solutions of chaotropes seems to be widely determined by preferential solvation. Between both chaotropes, larger preferential solvation of the overall protein surface has been attributed to the guanidinium ion (Courtenay, *et al.*, 2001; Schellman, 2003). Most certainly, dissimilarities in the capability to solvate the overall protein surface additionally explain differences in the action of the three denaturants. It is plausible that abrupt unfolding impelled by guanidine hydrochloride at intermediate and high concentrations was largely driven by preferential solvent interaction with the protein.

Drawing correlations between properties of chemical compounds (for instance, polarity, hydrophobicity, proton donor / acceptor ability, dielectric constant, hydration, molecule geometry and size) and their efficiencies for denaturing a protein is a hard task. Difficulties arise from the multiplicity of properties potentially contributing to the denaturing action, and from differences in relative magnitudes of properties of each chemical compound. Moreover, the general effect of a denaturant is believed to consist of a summation of effects, *viz.*, direct binding to the protein, modification of the water structure and excluded volume effect. By virtue of this, reflections described above should be taken as simple attempts to establish clear-cut associations between properties and denaturing power of the chemical compounds employed in this research, and should be taken with due care. Ascertainment of their validity demands heedful studies strategically engendered in order to, for instance, isolate contributions from each property.

A.1.1. *Resistance to Chemical Denaturation*

In general, extension of the period of exposure of porcine pepsin A to chaotropes from 1 h to 3 days resulted in accentuation of the degree of structural disarray, when the protein was incubated in mildly concentrated solutions of guanidine hydrochloride [see Figures 4.33(a) and 4.36(a)] or highly concentrated solutions of urea [see Figures 4.35(a) and 4.37(a)]. Still, from a wide look at the literature, the protein under study appears to be more protected against large-scale unfolding by these two powerful chaotropes in comparison with a large number of other proteins, including other monomeric proteins in their native or native-like conformational states, such as cutinases (Ternström, *et al.*, 2005), type II shikimate kinase from

Erwinia chrysanthemi (Cerasoli, *et al.*, 2002), or human serum albumin (Ahmad, *et al.*, 2005). Comparisons were made in terms of chaotrope concentrations required for triggering major unfolding, and took into account the length of incubation phases preceding measurement of structural properties. This finding prompted the search for unusual features in the protein under investigation.

Strong acidophilicity is an outstanding characteristic of porcine pepsin A. Critical determinants of high conformational stability of this protein at low pH have been identified. It possesses a high ratio of acidic to basic residues. There are only four basic residues, and they are buried or partially buried. His53 and Lys319 are localized at opposite sides and, hence, the mutual interaction between them does not affect conformational stability. Arg307 and Arg315 are neutralized by formation of ion pairs with Asp11 and Asp138, respectively. All in all, there is no significant electrostatic repulsion between positively charged residues in acidic media, in contrast to the majority of the proteins in which repulsion of positively charged moieties causes a decline in conformational stability in such environmental conditions. On the other hand, there are 43 acidic residues. Several of their carboxyl groups have abnormally low pK_a values due to atypical hydrogen-bonding environments, insomuch as they exhibit negative charges even in strongly acidic media (Andreeva, *et al.*, 1984; Fushinobu, *et al.*, 1998). Large excess of acidic residues over basic residues and unusually low pK_a values of some acidic residues, together with a phosphoryl group with a low pK_a covalently bound to Ser68, contribute to the low isoelectric point (lower than 1) determined for pepsin (Andreeva, *et al.*, 1984; Fruton, 1970). Aside from such exceptional property, excessive incidence of acidic residues at the protein surface (see Figure 5.1) is as well believed to contribute to acid stabilization of porcine pepsin A (Fushinobu, *et al.*, 1998). In fact, about 23 out of the total number of acidic residues were surmised to be exposed (*i. e.,* were estimated to have more than 50% of relative solvent accessible surface area), and noted to be dispersed over all protein surface.

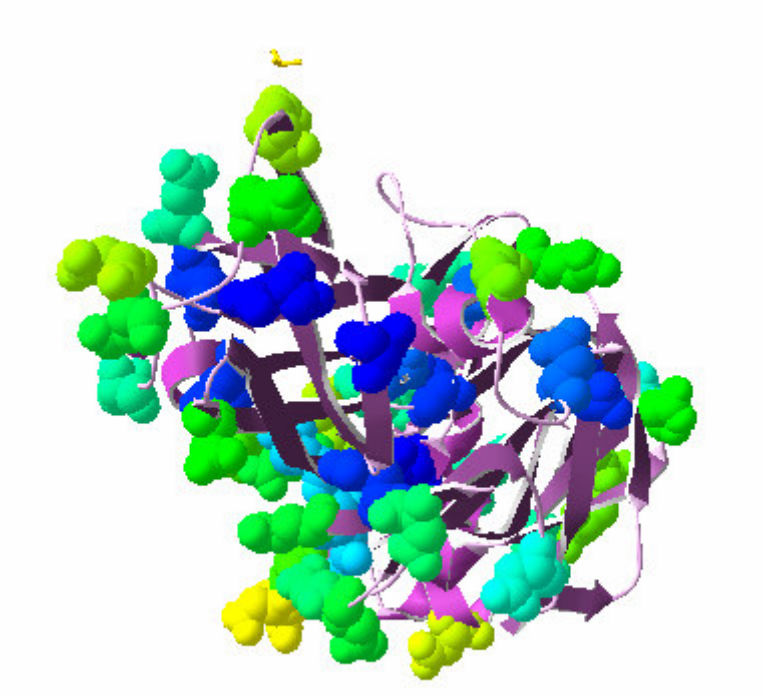

Figure 5.1 – Distribution of acidic residues in the three-dimensional structure of porcine pepsin A (PDB code *4pep*). The main chain trace is shown in cartoon format. The phosphoryl group is coloured in yellow. Acidic residues are coloured according to accessibility. The colour scheme includes spectral colours: on one end, dark blue stands for low relative surface accessibility; on the opposite end, red stands for relative surface accessibility equal to or greater than 75%. This figure was generated by means of the interface *Swiss-PdbViewer 3.7*.

In view of what was aforesaid, porcine pepsin A in aqueous medium at pH 3 is negatively charged. And, the distribution of exposed acidic residues is proposed to favour the occurrence of areas of strong hydration nearly all over the protein surface. This assumption mostly relies on the fact that the first hydration shell of negatively charged side chains exhibits higher density and two-fold longer residence times in relation to the first water layer of most other protein groups (Beck, *et al.*, 2003). Strong hydration of aspartic and glutamic residues around charged carboxyl groups could be related to strong partial negative charge on the carboxyl oxygen.

Different correlations presented in the preceding chapter and ongoing subsection reflect an apparent dependence of the natively folded conformational state of porcine pepsin A on its solvation shell, which is presumed to be dense and tightly held to the protein surface in consequence of considerations embodied in the previous paragraph. Compatibility between such dependence and an ancient suggestion for a major role of solvent-mediated hydrophobic interactions in the stabilization of the native protein structure (Perlmann, 1959) might be rationalized as follows. Hydrophobic interactions between non-polar groups within a globular protein have been claimed to result primarily from the pressure exercised by water molecules that do not penetrate inside the globule due to repulsion between hydrophobic moieties and solvent, rather than from a direct affinity between non-polar groups (Cooper, 1999; Privalov, 1996 and references therein). Enhanced hydration at the surface of native porcine pepsin A might intensify external pressure of the water, thereby

improving interactions between non-polar moieties in the protein interior. Sustainment of the natively ordered arrangement of the protein under study by a strong hydrophobic effect is further supported by the following pieces of evidence available in literature. Privalov and co-workers (1981) identified four main hydrophobic regions in pepsin molecule, which allegedly engage more internal than external contacts. Later, Andreeva and co-workers (1984) alluded to the existence of extensive, internal hydrophobic areas, with major implications for conformational stability of this protein.

Resistance to chemical denaturation might not arise from a single mechanism, but from several contributions. Besides a strong hydrophobic effect, other strategies might be involved in improved tolerance against large-scale unfolding by the classic chaotropes employed in this research. In all likelihood, a second consequence following from a dense hydration shell tightly held to the protein surface is the limitation in the access of these denaturants to the protein surface. Indeed, poor hydration of guanidinium ion and urea should mostly favour their interaction with weakly hydrated regions of the protein (Collins, 1995; Washabaugh & Collins, 1986). In the end, enhanced hydration might contribute to a strong hydrophobic effect and a limited access to the protein surface, which in turn might, at least partially, explain improved protection of porcine pepsin A against gross unfolding induced by classic chaotropes.

Analysis of experimental data obtained throughout this research for porcine pepsin A and comparable data determined for HIV-1 (Grant, *et al.*, 1992; Szeltner & Polgár, 1996) and SIV proteinases (Grant, *et al.*, 1992) confirms higher conformational stability of the monomeric as compared to the homodimeric aspartic proteinases. Urea concentrations in the range from 2.00 to 4.00 M are sufficient for inactivating and unfolding HIV-1 and SIV proteinases (Grant, *et al.*, 1992).

A.2. *Elevated Temperature*

Previous research on heat-induced denaturation of pepsin has mostly focused on lowly acidic, neutral and alkaline pH ranges (Dee, *et al.*, 2006; Privalov, *et al.*, 1981; Tello-Solís & Romero-Garcia, 2001). Herein, measurements of circular dichroic absorbance in ascending thermal gradients and differential scanning calorimetric analyses targeted the natively folded state (at pH 3). Results obtained suggest that the process of thermal denaturation of native porcine pepsin A did only involve two macrostates, the natively folded and the unfolded states (see section 1.C. '*Thermal Denaturation and Stability*' of the chapter IV).

A.2.1. *Thermal Stability*

In aqueous medium, thermal transition temperature of porcine pepsin A was 72.08 – 72.72ºC (depending on the ionic strength and the nature of salts in aqueous media) as determined by differential scanning calorimetry (see Figures 4.15, 4.17 and 4.31). Given that heat-induced transitions of proteins classified as thermophilic generally have midpoints within the 70 – 100ºC interval (Torrez, *et al.*, 2003), the

protein under study was concluded to be rather thermostable.

Knowledge on effective strategies underlying thermostabilization has largely benefited from comparative theoretical and experimental investigations on comprehensive sets of hyperthermophilic and / or thermophilic proteins, and their mesophilic homologues (Davies, *et al.*, 1993; Kumar, *et al.*, 2000; Sadeghi, *et al.*, 2006; Sterner & Liebl, 2001; Vieille & Zeikus, 2001; Zhou, 2002). Nonetheless, a unifying picture of the physical and molecular basis of thermal stability has not yet been drawn. Improved hydrophobic interactions are included in the list of contributory factors to thermostabilization (Sterner & Liebl, 2001; Vieille & Zeikus, 2001). This might well consist of one among other sources of thermal stability of porcine pepsin A, taking into account the proposal for a strong hydrophobic effect previously justified.

B. *Denatured States*

Each type of perturbant alters the balance of stabilizing forces within a protein in a different way. And, thus, it is intuitively expected that a protein will not denature to reference states, and will adopt a variety of non-native states with distinct properties. The conformational features and the degree of structural disarray in each denatured state of a given protein have been recognized to be largely dependent on environmental conditions and, to some extent, on its amino acid sequence (Dill & Shortle, 1991; Nolting, *et al.*, 1997; Pace, *et al.*, 1990a; Royer, *et al.*, 1993; Schellman, 2003; Whittington, *et al.*, 2005). Accordingly, induction of denaturation of porcine pepsin A by different methods yielded a set of structurally diverse non-native states. A summary of gross structural properties of unfolded and partially unfolded states is presented in the Table 5.1.

Table 5.1 – Structural properties of unfolded and partially unfolded states of porcine pepsin A. In order to facilitate comparisons, values of circular dichroic signal of the native and the final acetonitrile-denatured states were normalized to 1.00 and 0.00, respectively, and values pertaining to other conformational states were scaled accordingly.

Denatured States (Conditions of Incubation)	**CD at 216 nm**	**CD at 290 nm**
Native State (10 mM Sodium Acetate at pH 3, 1 h at 25ºC)	1.00	1.00
Acetonitrile - Denatured State (15.39 M ACN, 1 h at 25ºC)	0.00	0.00
Final Guanidine Hydrochloride - Denatured State (7.22 M GdnHCl, 1 h at 25ºC)	0.38	0.05
Intermediary Guanidine Hydrochloride - Denatured State (5.89 M GdnHCl, 3 days at 25ºC)	0.35	0.38
Final Guanidine Hydrochloride - Denatured State (7.22 M GdnHCl, 3 days at 25ºC)	0.40	0.09
Partially Unfolded State in Non - Aqueous Mixtures of GdnHCl (5.70 M ACN + 4.54 M GdnHCl, 1 h at 25ºC)	0.50	#
Heat - Denatured State in Aqueous Medium (10 mM Sodium Acetate at pH 3, 1 h at 25ºC)	0.37	#
Heat - Denatured State at Low ACN Contents (1.90 M ACN, 1 h at 25ºC)	0.36	#
Heat - Denatured State at Intermediary ACN Contents (5.70 M ACN, 1 h at 25ºC)	0.19	#

The end-point of acetonitrile-mediated unfolding of porcine pepsin A consisted of an unstructured conformational state, whose native secondary and tertiary arrangements were globally disrupted. However, protein denaturation is frequently not accompanied by complete unfolding. This was noticed for porcine pepsin A when guanidine hydrochloride and temperature were used as destructuring agents. Final products of order-to-disorder transitions brought about by addition of increasing amounts of charged chaotrope to samples of native porcine pepsin A, and by a raise in temperature of samples of protein in aqueous medium or in a binary aqueous mixture of acetonitrile at 1.90 M corresponded to unfolded states with close residual contents of secondary structural elements. Addition of 5.70 M acetonitrile to aqueous medium lessened the degree of organization in the remaining secondary structure of the final heat-denatured state. Unfolded and partially unfolded states identified along the pathway of guanidine hydrochloride - induced denaturation exhibited comparable backbone order. Nonetheless, these denatured states differed mainly in terms of tertiary structure, judging by residual rigidity of aromatic side chains in partially unfolded states which was largely lost in unfolded states.

One can find descriptions of other non-native states of pepsin in literature. The one best characterized is the partially unfolded state sampled by this protein at mildly alkaline pH (the so-called, I_p *state*). It conserves substantial amount of ordered structural units and tertiary structural organization (Campos & Sancho, 2003; Dee, *et al.*, 2006 ; Konno, *et al.*, 2000). The N-terminal domain preserves some degree,

albeit low, of non-random structure. The C-terminal domain undergoes a less extensive loss of native structural arrangement in comparison with the N-terminal domain, retaining a compact organization. Both domains contain non-native structure (Kamatari, *et al.*, 2003; Konno, *et al.*, 2000). Pepsin loses its residual secondary and tertiary structure in the 11 – 12 pH interval in a cooperative manner (Konno, *et al.*, 2000).

Lately, biological implications of non-native states in issues, such as protein folding (Schwarzinger, *et al.*, 2002; Smith, *et al.*, 1996; Uversky, *et al.*, 1999), chaperone binding (Sigler, *et al.*, 1998), transportation (Bychkova, *et al.*, 1998), stability (Smith, *et al.*, 1996; Uversky, *et al.*, 1999) and fine-tuning of function (Robic, *et al.*, 2003) have been discussed. In respect of pepsin, Campos and Sancho (2003) have detected an equilibrium intermediary conformational state in the pH range from 4 to 6.5 (as abovementioned), which was implied in the transport of the protein to the stomach lumen.

Neither significance nor nature (native or non-native) or localization of remaining structure within denatured states listed in Table 5.1 can be inferred from data obtained within the scope of this research. Drawing conclusions in this regard shall demand application of complementary approaches.

C. *Small-Scale Destabilization Prior to Large-Scale Denaturation*

C.1. *Chemical Compounds*

Besides structural properties, the impact in hydrodynamic, biophysical and functional properties of porcine pepsin A ensuing from 1 h - exposure to denaturants at varying molarities was as well examined. It was found that modifications in Stokes radius, thermal stability and enzymatic activity of the protein generally occurred or started to occur before the onset of large-scale unfolding.

In sets of experimental conditions (length of incubation phase and denaturant concentration) which did not favour major conformational changes as tracked by spectroscopic methods, size exclusion chromatographic analyzes of porcine pepsin A pointed to: (i) subtle debilitation of its hydration shell induced by acetonitrile (see Figure 4.14); and (ii) local swelling and / or unfolding induced by chaotropic agents (see Figure 4.28). These variations were concomitant with decreasing trends in thermal transition temperature (see Figures 4.17 and 4.30), which are taken to reflect declines in conformational stability of porcine pepsin A. Indeed, thermal stability is frequently regarded as a practical measure of thermodynamic conformational stability. Acetonitrile and guanidine hydrochloride at pre-transitional concentrations were found to induce greater destabilizing effects than equimolar urea. This observation is in accordance with the higher efficiency of the former denaturants to trigger gross unfolding of porcine pepsin A as compared to the latter.

Events previously reported were accompanied by decreases in enzymatic activity (see Figures 4.20 and 4.32). Minute conformational changes in regions proximal to or at the catalytic apparatus induced by denaturants were presumed to have contributed to deceleration in the rate of hydrolysis of the peptide bond, *p* – nitro – Phe – Nle, catalyzed by porcine pepsin A, prior to the onset of large-scale unfolding. Interestingly,

total or nearly total abolishment of peptidolytic activity was detected when porcine pepsin A still sampled a native-like conformational state in the presence of acetonitrile and urea, as manifested in structural data; whereas guanidine hydrochloride did only cause full inactivation of the enzyme when it achieved the end-point state of the conformational transition brought about by this chaotrope. These observations prompted the search for additional explanations for modifications in functional properties provoked, in particular, by the organic solvent and urea. It was hypothesized that interferences with the hydration of the microenvironment of the catalytic apparatus could partly originate lower hydrolytic rates. As recurrently mentioned, acetonitrile is able to strip water from the solvation sphere of proteins, and it is a poor substitute for water as a solvating agent. Conversely, urea and water appear to readily replace for each other in solution. Yet, it is likely that the roles of water in the catalytic mechanism cannot be fulfilled by the chaotrope. Ultimately, differences in the activity profiles obtained for porcine pepsin A in the presence of the three denaturants might be related with differences in their affinity to the catalytic apparatus and / or other regions in the protein with a role in maintaining the adequate geometry and stability of the catalytic apparatus.

C.2. *Transition from Mildly Acidic to Lowly Alkaline pH*

Influence of proton concentration on porcine pepsin A has been intensively investigated. On reviewing open literature pertaining to this issue, it seems that some characteristics of pH titration profiles are dependent on experimental variables. For instance, as abovementioned, Campos and Sancho (2003) succeeded in identifying an intermediary conformational state in the pH interval of 4.0 – 6.5 under equilibrium conditions (Campos & Sancho, 2003). On the other hand, in a few studies whose authors do not refer to exposure to experimental conditions for a fixed period of time prior to measurements, pepsin was considered to be in its natively folded state at pH 5.3 – 5.6 (Dee, *et al.*, 2006; Kamatari, *et al.*, 2003; Konno, *et al.*, 2000). Nonetheless, all these studies have seemingly agreed that a raise in environmental pH to values in the range from 6/6.5 to 7 spontaneously triggers a disordering conformational transition in pepsin (Campos & Sancho, 2003; Kamatari, *et al.*, 2003; Konno, *et al.*, 2000). On the whole, conformational instability in neutral and alkaline solutions has been explained by electrostatic repulsion among negatively charged side chains (Lin, *et al.*, 1993; Tanaka & Yada, 2001). As aforesaid, acidic residues are highly incident in this protein. The N-terminal domain has been surmised to be the trigger of denaturation at neutral-alkaline pH (Tanaka & Yada, 2001). In particular, it was proposed that carboxyl groups of Glu4, Asp11, Glu13, Asp118 and Asp159 are in a deprotonated state in these pH ranges (Lin, *et al.*, 1993). Proton release from side chains is followed by conformational changes (Favilla, *et al.*, 1997), so that these protein groups become more accessible to and stabilized in aqueous environment (Lin, *et al.*, 1993).

Even though the conformational transition onset at neutral pH values results in a partially unfolded state as described earlier, it displays a lesser degree of tertiary structural organization and decreased contents in β - sheets, and it is more extended in relation to the native state (Aoki, *et al.*, 1997; Campos & Sancho, 2003; Dee, *et al.*, 2006; Konno, *et al.*, 2000).

In referring to a few experiments on the impact of variations in pH on the hydrodynamic behaviour and thermal stability of porcine pepsin A performed within the scope of this project, results obtained after a 1 h - incubation phase indicated that the protein became expanded, judging by increased Stokes radius at pH 5.5 and 6 in comparison with pH 3 (see section 2.B. '*Hydrodynamic Behaviour*' of the chapter IV). This finding is compatible with conclusions obtained by Dee and associates (2006), according to which alkaline-denatured conformations of pepsin A tend to be elongated and nearly cylindrical. Moreover, conformational stability underwent an abrupt decrease from pH 4 to 6, as analyzed by differential scanning calorimetry (see Figure 4.31). It follows therefrom that an increase in pH to values lower than those required for the onset of major denaturation could destabilize the protein.

Heating of samples of porcine pepsin A at pH 7 did not result in any observable conformational transition, which is compatible with significant exposure of hydrophobic surfaces as suggested from adsorption of the protein to the matrix of the size exclusion chromatographic column and the onset of the well-known conformational transition at neutral pH.

These results are at odds with analogous data obtained by other authors who, without referring to an incubation phase prior to their assays, have reported preservation of the native hydrodynamic radius until about pH 6 (Campos & Sancho, 2003), and heat-induced conformational transitions even at pH 8 (Dee, *et al.*, 2006). In principle, these discrepancies sustain time-dependence of effects of pH on porcine pepsin A.

2. *Enhancement of Order and Apparent Stabilization at Global and Subglobal Levels of Porcine Pepsin A by Chaotropes*

Even though situations have been reported in which the presence of acetonitrile induced preferential hydration of a protein, leading to formation of interpeptide hydrogen bonds and an increase in helicity (Gekko, *et al.*, 1998), this organic solvent appears to have dominantly acted as a destructuring agent on porcine pepsin A in the chosen experimental setup. Conversely, chaotropes were able to improve backbone order, to inhibit unfolding and to enhance packing before the onset of large-scale denaturation.

Incubation in lowly and highly concentrated aqueous solutions of urea for 1 h led to a small improvement of the degree of secondary structural organization of porcine pepsin A [see Figure 4.23(a, c)]. Notwithstanding the molecular basis of structure-promoting action of urea is still poorly understood; the observed effects were assigned to a raise in backbone rigidity upon favourable interaction of the neutral compound. This phenomenon was not, however, accompanied by an increase in overall conformational stability as gauged by differential scanning calorimetry (see Figure 4.30).

Spectroscopic data obtained at the third day of exposure to guanidine hydrochloride and urea provide evidence for other atypical protein responses to these chaotropes. After being incubated for 3 days in denaturant-free aqueous medium at 25ºC, porcine pepsin A was noted to undergo small-scale unfolding. Nevertheless, addition of the charged or the uncharged denaturant at low molarities to incubation medium had an apparent stabilizing effect, since disruption of secondary structure was totally or partially hampered

[see Figures 4.33(a, b) and 4.35(a, b)]. Furthermore, gains [see Figure 4.37(b)] or regains [see Figure 4.36(a)] in order of local surroundings of aromatic side chains were also detected before gross unfolding. These phenomena are interpretable in terms of entropic effects of both chaotropes. It is possible that mechanisms of electrostatic character were as well involved in the apparent stabilizing action of guanidine hydrochloride. Except for the resistance against small-scale unfolding seen at the third day of submission to 1.90 M guanidine hydrochloride, all other situations of apparent stabilization of porcine pepsin A by chaotropic agents did only occur at a subglobal level.

CHAPTER VI

CONCLUSIONS & FUTURE DIRECTIONS

Herein, general conclusions derived from the findings of this research, together with proposals for new directions in the study of denaturation and conformational stability of porcine pepsin A, will be pinpointed.

Spectroscopic data on conformational transitions and non-native states suggest that diverse denaturing conditions afforded different scenarios of unfolding of natively folded and biologically active porcine pepsin A. Denaturation profiles determined after protein incubation in binary aqueous mixtures of acetonitrile at increasing molarities for 1 h revealed non-cooperative destruction of secondary and tertiary structures, which culminated in a largely unstructured state.

In a comparable experimental setup, porcine pepsin A achieved an unfolded state, in guanidine hydrochloride - rich aqueous solutions, which still exhibited some secondary structural elements. On long exposure to the charged denaturant, the contents of β-structure in this disordered state were essentially maintained. And, a stable, partially unfolded state could be identified in mildly concentrated solutions of chaotrope. The aromatic side chains displayed lower conformational mobility in the intermediate as compared to the unfolded state, suggesting the preservation of a few tertiary interactions in the former state *vis-à-vis* the trivial tertiary structure in the latter.

Preliminary results were obtained for the conjugate action of acetonitrile and guanidine hydrochloride on porcine pepsin A. A few cases of cumulative disorder were detected. In the most outstanding case, the conjunct effect of the two denaturants at intermediary molarities far outweighed the sum of their isolated actions, corresponding to a reduction of about half of the typical amount of β-sheets.

Urea-assisted unfolding revealed to be a slow-rate process. At the third day of submission to uncharged denaturant at a near-saturating concentration (8.79 M), porcine pepsin A still contained about half of its native contents of secondary structural units, and its aromatic side chains still occupied considerably fixed positions.

It appears that the order-to-disorder transition of native porcine pepsin A produced by raising the

temperature of the protein-solvent system did only involve two conformational macrostates, the folded and the unfolded states. The heat-denatured state possessed a significant amount of β-sheets and α-helices. Such conformational state became closer to a random coil - like state when protein samples had intermediate contents of acetonitrile.

Elucidation of reasons underlying accumulation of partially unfolded states and persistence of non-random structure within unfolded states should benefit from a more extensive analysis of denatured states, which would demand application of alternative techniques, such as limited proteolysis, small-angle X-ray scattering or nuclear magnetic resonance spectroscopy.

In general, data acquired by application of methods responsive to changes in hydrodynamic, biophysical and functional properties of porcine pepsin A provoked by addition of denaturants and raises in pH were useful in complementing structural evidence obtained from spectroscopic measurements. They pointed to small-scale destabilization at pre-unfolding conditions, and conveyed differences in the mechanisms of action of various perturbants.

A possible correlation between properties of denaturing agents and their capability to cause order-to-disorder transitions was drawn. It was suggested that the demand for greater concentrations of urea and longer periods of exposure to this denaturant to prompt major unfolding of porcine pepsin A, in comparison with guanidine hydrochloride and acetonitrile, could be partially rationalized by higher potential of the uncharged chaotrope for establishing hydrogen bonds with water molecules and substitute for them in the water network. And, hence, urea would better compensate for loss of water molecules from the protein hydration sphere than any other of the two denaturants. Obviously, other issues should be called into play when trying to explain mechanisms of action of these denaturants, namely, solvent quality. It is conceivable that preferential solvation by guanidinium ion largely determined abrupt unfolding of porcine pepsin A at intermediary and high molarities of charged denaturant. Detailed investigations on the interactions of denaturants with the protein and on their effects on the protein hydration sphere would be needed, in order to establish whether these proposals are valid. Powerful techniques like, for instance, molecular dynamics simulations would be required.

The hypothesis for a relationship between the ability of a denaturant to replace for water molecules in hydration networks and its denaturing power presumes an important role of solvation structures on the conformational stability of porcine pepsin A, which was corroborated by experimental evidence previously reported. In view of the excess of negatively charged groups in the surface of this protein (one peculiar characteristic partially responsible for its high acidostability), the solvation shell was envisaged to be dense and tightly held to the protein. This feature, together with extensive internal hydrophobic regions, was proposed to contribute to a strong hydrophobic effect, which in turn might play a role in coping with some harsh environments. Indeed, under native-like conditions, porcine pepsin A was found to exhibit thermal stability comparable to that of thermophilic proteins, and enhanced tolerance against large-scale unfolding by classic chaotropes. Stabilization strategies do not necessarily depend on a specific mechanism. An additional consequence following from strong hydration might be limitation of access to the protein surface by chaotropes, which could delay major structural disarray. In order to appraise the significance of a high

negative electrostatic potential at the surface of porcine pepsin A, it may prove useful adopting mutagenesis approaches for modulating the contents of surface acidic residues, or performing comparative studies with chymosin, a structural neighbour of the protein under study with a lower number of exposed acidic residues.

It is well-established that porcine pepsin A is an acidophilic protein. Results obtained in this research showed that its capability to tolerate severe environmental conditions is not restricted to adaptation to extremely acidic media. Aside from high thermal stability, this protein has also revealed somewhat unusual resistance against large-scale unfolding induced by two powerful denaturants, guanidine hydrochloride and urea. It would be interesting to investigate whether or not this protein is able to preserve its natively ordered arrangement under the effect of alternative structural perturbants like, for instance, strong detergents or other organic solvents and chaotropes.

Simultaneous occurrence of these peculiar properties in one protein is not very common. In view of this, porcine pepsin A and other proteins capable of tolerating environmental severity of various kinds like, for instance, endo-β-glucanases (Chiaraluce, *et al.*, 2002; Huang, *et al.*, 2005; Koutsopoulos, *et al.*, 2005), might become interesting tools for searching for molecular strategies of general adaptation to extreme conditions.

Prior to major unfolding, non-conventional effects of chaotropes on proteins, entailing structurization and apparent stabilization at global and subglobal levels, were noticed mainly upon prolonged incubation periods. Enhanced backbone order followed as a consequence of submitting natively ordered porcine pepsin A to urea for a short period of time. A notable fact is the observation of such behaviour even at chaotrope molarities as high as 8.00 M. On extending the incubation phase to 3 days, one could notice that the presence of guanidine hydrochloride and urea totally or partially hampered destruction of secondary structure and promoted order in the arrangement of aromatic side chains. Occurrence of apparent stabilization at global and subglobal levels was attributed to entropic effects of both chaotropic agents. The apparent stabilizing action of guanidine hydrochloride was presumed to additionally entail salt effects. Sodium chloride could be used in order to ascertain and appraise the significance of processes of electrostatic character in stabilization of, not only native-like forms, but also partially unfolded states.

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